

Chips with everything

THE science of human nutrition in Britain has fallen on hard times. Of that, even the practitioners themselves seem to agree. In the last war the nutritionist played a major and acknowledged role in keeping the public healthy (some would say healthier than ever before or since). These days scientists turn their noses up at the thought of becoming nutritionists and the public gets little or no advice on what constitutes a balanced diet—apart from keeping cigarettes out of it. The situation seems to be worse, if anything, elsewhere in Europe.

Nutrition is not an easy subject to define, as it includes so many aspects of other disciplines. Nevertheless it may be helpful to make subdivisions such as

- (i) clinical nutritionists dealing with individual cases and counselling at the individual level,
- (ii) scientific nutritionists working, say, on vitamins, fortification or dietary assessment techniques,
- (iii) epidemiological nutritionists handling the statistics of large populations and
- (iv) public nutritionists working either with government or the food industry on monitoring and information.

Who becomes a nutritionist? It is here that the problems start. There is no prescribed way into the field, but a broad biological education, probably with a medical background, is desirable. However, the job lacks kudos and the attraction of the microscopic and sub-microscopic in biology draws away many talented students from study of the whole man. In medical schools, nutrition is about on a par with chiropody in appeal to students, and, for lack of good teachers, has often been introduced incidentally in other courses. A clinical nutritionist will often find himself a different title such as endocrinologist. As a result, those in nutritional departments of universities find it extraordinarily difficult to recruit high-calibre postgraduates, who regard the subject as soft and indefinite.

There is also a problem in the student's perception of a career structure. There are several senior posts in nutrition which will fall vacant in the next few years and there will be difficulty, it is said, in filling these, as there are insufficient middle level positions. Students see the lack of obvious upward steps from junior posts and they leave for better guaranteed careers.

Nutrition is very obviously a subject where the interplay between universities, industry and the public health services should be strong, but the deficiencies in career prospects in the academic sector have not been made up for by an adequacy of challenging positions elsewhere. The food industry does not employ many nutritionists (in contrast with the animal feed industry with its obvious interests in fatter beasts—the whole man may not be a proper study of mankind, but the whole pig certainly is). Those that industry does use are frequently engaged simply in ensuring the accuracy of labels or

approving advertisements. Those in public service too often find themselves engaged in routine activities, whilst there are large public health educational issues which go untouched.

Obesity is a case in point. Many believe that this is the major nutritional problem facing the Western world today, not only in itself, but for the many disorders which spring from it. Yet at present next to nothing is being done by public health authorities either in prevention or cure. Nutritional counselling even of the simplest kind barely exists. And for health clubs or dieting systems claiming to reduce weight safely there is no public supervision. This at a time when the choice of foods available to the consumer is large and there is a demand for information on good eating habits.

There are, fortunately, signs of change. The recent establishment of two Royal Society Sainsbury Fellowships should go some way towards producing better prospects for career advancement. Further, there is an indication that a new generation of students has a greater interest in multi-disciplinary people-oriented biology. And the new administrative areas of the National Health Service should each have a 'community dietician'.

Much remains to be done, however, in the field of health education. The responsibility is ultimately the government's and the duty is to make the public aware of the elementary principles of sound nutrition so that more sophisticated choices can be made when buying food. It has long been a British principle to assume that intuition is a good substitute for understanding, and therefore that detailed information about the nutritional properties of food is unnecessary. It is time that such an attitude was superseded.

The results of better nutritional counselling would be unspectacular, and there would be inevitable doubts that a long-term educational programme would have any more impact than have anti-smoking campaigns. This should not deter government from taking a few inexpensive steps in the right direction. They would at least stimulate a greater commitment to nutritional studies by the food industry. In turn, this might breathe more life into research in the universities.

100 years ago



A PETITION signed by twenty-six Professors in the Universities of Scotland has been presented to the Prime Minister, calling his attention to the treatment of the ladies admitted to matriculate as students of medicine in the University of Edinburgh, and afterwards refused the right to graduate, and urging the Government to take the whole subject of the University education of women into consideration, with the view of devising a remedy for the present anomalies.

From *Nature*, 10, 16, May 7, 1874.



Cold comfort from Friends

In a recent letter to the Secretary of State for Energy, Mr Eric Varley, Friends of the Earth (a charitable organisation committed to the conservation, restoration and rational use of the ecosphere) drew attention to the energy savings possible through improved insulation standards and urged that he encourage the introduction of measures which would put an end to unnecessary waste. Here Christine Thomas, a researcher with Friends of the Earth, describes the sort of savings which might be made with a policy for insulation.

Friends of the Earth recently spent a Saturday insulating the roofs of the homes of some 40 old age pensioners throughout the country. They did so in order to draw public attention to the waste caused by heat loss through poorly insulated buildings—an estimated 75% of the energy consumed for space heating in homes insulated to present standards.

Improved insulation can drastically cut this loss; domestic fuel consumption for heating could be reduced by as much as half while still maintaining the same degree of comfort, or indeed improving it. Such savings matter most for those on small or fixed incomes—in particular the elderly, whose health and incomes can least withstand excessive fuel bills. According to a recent national study about 6 million old people are living in rooms clearly too cold for comfort, between 500,000 and 700,000 of them having unacceptably low body temperatures: clearly candidates for hypothermia.

It was facts like these that prompted the Friends to join with Age Concern (the National Old People's Welfare Council) in a practical gesture to demonstrate the ease with which insulation standards could be improved. Laying glass fibre or mineral wool in an attic costs perhaps £20, and offers at present prices a fuel cost saving of some 16% a year in the average semidetached house. Cavity walls can be filled with a variety of foams and mineral substances; solid walls can be faced with insulating board; windows can be double glazed. An average investment of £300 would cover improvements to the roof, walls and windows, together with draught exclusion, cutting fuel consumption by almost a half. Such a sum can be recouped in less than 10 years by the concomitant savings. Double glazing, however, forms a disproportionately large part of this cost, by comparison with the heat loss it can prevent. Something like a reduction of 35% in fuel consumption can be achieved by increasing roof and wall insulation alone at a cost of around £100.

Since domestic heating accounts for 20% of the total energy consumed in Britain, the potential national saving would be about a tenth of the present amount of energy used, the equivalent of nearly 11 million tonnes of oil each year. Calculations show that the power demand that would be eliminated by such a reduction is some 38,000 MW, equivalent to the combined output of some 50 large nuclear reactors. It would, of course, be expensive to insulate existing housing to these standards; a sum of the order of £5,000 million would be required. This, however, compares favourably, with for instance, the capital cost of the power stations that would otherwise be needed: at least £10,000 million. Thus there could be a saving of more than £5,000 million in the long run, not to mention savings in environmental and social costs associated with the installation of large scale generating plant.

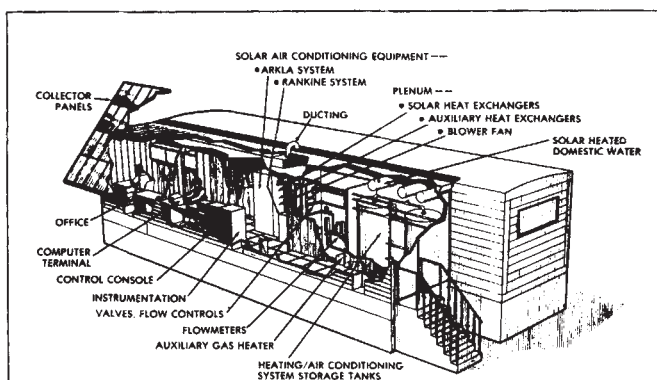
It would clearly be far cheaper to insulate all homes fully than to build unnecessary power stations purely to supply

heat to waste. Furthermore, by thus relieving some of the pressure on present energy supplies, a very important breathing space would be gained: time to look closely at genuine energy requirements and how they can best be fulfilled, time to develop alternative sources of power, sustainable and safer sources.

The improvement of insulation standards is an area of energy conservation readily amenable to legislative encouragement. Minimum levels for new dwellings are set in the Building Regulations by stipulating the maximum values of U (a measure of the conductivity) permitted. The regulations are at present made under the Public Health Act, which stipulates that they can only be made with specific reference to considerations of health and safety. There is argument as to whether present standards are adequate even for this limited purpose, and proposals for amendments are at present being considered. This, however, seems as far as the present regulations can be pushed.

In order that higher standards, justifiable in terms of energy savings, can be set, the scope of the Building Regulations will have to be widened. This was discussed under the Heath administration when the Department of the Environment was promoting Part III of the Health and Safety at Work etc. Bill, which would have empowered the Secretary of State to make regulations specifically "furthering the conservation of fuel and power" (III, 61, 2, b).

At the discretion of a Local Council, Home Improvement Grants may at present be given for some upgrading of insulation. But the position is not clear; people are unaware of the possibility and many councils are reluctant to give such grants. Making insulation the subject of a Standard Grant would dramatically improve the availability of money; and, together with a positive campaign of public education to ensure that people took advantage of the scheme, would go a long way towards improving standards.



A TRANSPORTABLE laboratory to collect data on incoming solar energy and to carry out advanced research on solar energy heating and cooling systems under widely varying climatic conditions across the United States is completing tests in the Washington, D.C. area before beginning active experiments. The laboratory, housed in two large trailers, was taken to the Washington area for calibration and evaluation of its experimental systems and weather instrumentation at the National Bureau of Standards at Gaithersburg, Maryland. A major purpose of the research with the laboratory will be to obtain correlation of solar energy systems with local requirements which vary widely over the United States. In line with this purpose, local groups of designers, architects, building contractors, and zoning and building code officials in communities along its route will visit the laboratory to exchange information with researchers on specific needs in adapting such systems for use in their localities.

international news

AFTER seven months of junta rule in Chile academic life is stagnating. On the morning of the Allende government's overthrow in September the military occupied all of the country's nine universities, installed their own representatives in place of the deposed rectors, ordered mass suspensions of students and staff alike, and began a process of 'cleansing'. The result of this process is that those members of the academic community who avoided execution, torture or imprisonment, and who have not managed to join the thousands who fled the country, remain at the universities in puppet roles organising research according to the junta's decree, teaching what the junta thinks fit to be taught. If they were unfortunate enough to be working in the social sciences their chances of ever again doing academic work in their own country look remote. Even the Christian Democrats who balked at Allende's politics are now coming under fire, and as if the initial physical brutalities were not a sufficient token of the junta's intentions, Chile is being treated to a spectacle which makes clear its new rulers' attitudes to intellectual life: the burning of books.

Immediately after the coup the new regime closed most faculties and departments in the social sciences, history and philosophy. In the University of Concepcion 6,000 of the 16,000 students and hundreds of academics were suspended; the eastern campus of the University of Chile in Santiago was closed and over 8,000 students had their studies cut short, while 4,500 students and 100 academics in the university's school of fine art, music and architecture were suspended.

The Technical University in Santiago, where there was a massacre of students the day of the coup, has been changed into a "military polytechnic" and all of the students and teachers (about 20,000 students in all) have been expelled. All adult education programmes have been stopped. This puts an end to the educational schemes for workers that were carried out under the Popular Unity Government. It is estimated that 5,000 members of the Chilean academic community have fled to Mexico, and 1,000 to France, while smaller groups have escaped through foreign embassies to other European countries and to North and South America.

In Britain, where refugees had been welcomed with open arms from Czecho-

Where freedom is an academic issue

slovakia in 1968 and Hungary a decade earlier, there was official reluctance to mount a welcoming party for exiles who were themselves communist, or at least capable of living and working without complaint under a communist system. The removal of the Conservative government (one of the first to recognise the junta) in theory makes the way easier for Chilean academics looking for a base to re-establish their careers, but in fact only a handful have so far been accepted: 19 postgraduate students, two undergraduates and 14 visiting and research fellows.

An educational charity, the World University Service has joined with a pressure group called Academics for Chile in raising funds for displaced academics, and together they have produced more than £20,000 and pressed the British government for a contribution. The official response is that already £100,000 is being paid out in training and technical assistance to more than 70 Chileans, most of whom were in the country at the time of the coup, when aid was available on a government to government basis. The Department of Overseas Development is thought to be looking for new ways of continuing this aid now that a socialist government is in the driving seat.

Meanwhile, accounts of the average non-political academic's predicament at the time of the coup and since can be had at first hand in almost any university town in Europe. One such academic who left Chile after repeated harassment by the military and is now studying at a British university, recalled his experience in the following terms (his name is withheld because of his fears for relatives remaining in Chile):

"When the military government took over one of their first targets was the universities. On the day of the coup, the military occupied all nine universities in Chile and the Technical University in Santiago was bombed. The junta considered the universities the main focus of the social change that had swept the country since President Allende's government had come to power in 1970, and they seized power to put a stop to it.

"Their attention was especially concentrated on departments of economics, sociology, anthropology and history as these had been in the forefront of social change, with increasing emphasis being placed in the last few years on social research and educational projects in the shanty towns and amongst the workers and with increasing numbers of workers being given grants to study at the universities.

"After the coup the new government imprisoned many intellectuals even from the right-wing Catholic University in Santiago, and many are reported to have been tortured or executed. The rectors of all the universities were replaced by members of the armed services, and special 'prosecutors' were assigned to each department to hear information and charges placed against students and lecturers by informers.

"Many thousands of students and lecturers suspected of Marxist or socialist ideas who had escaped imprisonment were sacked, and in March all lecturers had to resign and reapply for their jobs. The appointments boards are appointed by the military rectors and the case of those lecturers sacked or refused reappointment on political grounds is grave. There is at present little hope of them being able to find other jobs in Chile, even as manual workers, as employment contracts are strictly scrutinised by the military authorities and no-one who has lost his job for political reasons can be re-employed.

"Whole university departments have been closed down, and I have heard of one department of social sciences where the whole department was imprisoned for publishing research on conditions in the shanty towns and in the country areas.

"As for myself, I was taken from my house by soldiers with no charges being made, and taken to the army barracks. There I was stood against the wall, guarded by four soldiers, for several hours before being interrogated. Already I found, the army had collected all my personal details and previous publications and activities, probably I thought from informers. I was accused of being a Marxist on the basis of my writings, but my interrogators obviously had no idea what Marxist philosophy really consisted of. I found it impossible to put up a legal and rational defence as the whole legal and moral basis of action had been overturned

and actions and writings which had been previously considered perfectly legal were now retrospectively considered illegal, such as not taking part in the anti-Allende demonstrations and strikes before the coup.

"I was interrogated ten times in all, and eventually I left the country rather than risk the alternatives of imprisonment, suspension or of being allowed to continue teaching only courses approved by the government.

"These restrictions in education have reached down as far as the primary schools, and in my opinion the military government is trying to turn the clock back in their educational policies by stopping grants for workers to study at the universities and by strictly controlling the matter and methods of teaching. The restrictions now being placed on freedom of speech are becoming intolerable to most intellectuals, even to those Christian Democrats who opposed Allende and had welcomed the coup."

The reference in this account to the overturning of the legal and moral basis of action is echoed in a further report from Dr H. M. Gerschenfeld, a signatory to a letter about the plight of Chilean academics published in *Nature* in March. According to the military government's Law no 111, suppressing the legal governing bodies of universities, 34 professors of the University of Valdivia were expelled without right of defence, says Dr Gerschenfeld. In some cases the professors were offered the alternative of resigning, and if they refused to do so, they were expelled.

Among more serious casualties of the new order were Professors Merio Ramirez (Education) and Jorge Pena (Music), shot at La Serena; Dr Enrique Paris, secretary of the University of Santiago, died while undergoing torture; Professor Kirkberg, rector of the Technological University of Santiago, imprisoned on Dawson Island.

As for institutions which have suffered under the junta, Dr Gerschenfeld writes as follows:

"The destruction of the departments of psychology and sociology occurred in essentially all of the universities in the country. Even internationally supported schools such as the well known Facultad Latino-americana de Ciencias Sociales (FLACSO) was closed. The Institute of National Studies (CEREN) of the Universidad Catolica at Santiago was also dismantled. Seventy to 100% of the faculty members in the departments of geography, literature, fine arts, history and geology were removed from their positions in Santiago.

"The authorities of the universities who were nominated by the military government also expelled a large number of students on the grounds of sympathising with the government of Dr Allende. The data are fragmentary,

but the examples are indicative: for instance, of the 254 first year students of the Science School at Santiago, 174 were expelled, at Concepcion, of 70 students in the last year of the medical school, only 10 were readmitted after a selection on political grounds.

"The number of scientists, teachers, and professional people who are leaving Chile is so great that the military junta has become alarmed. There have been rumours in the last month that the government will impose a tax of 3,000 to 5,000 American dollars to each person having a university degree who wants to leave the country, even if they have been expelled from their jobs at the university or elsewhere. We could not confirm this directly, but the appeals for help from our Chilean colleagues mention this menace and urge the European scientific community to do something for them."

An idea of the lengths to which the military government will go in order to interfere with the career of an academic whom they consider hostile is illustrated by the experience of Juan Rada, who at the time of the coup was a third year student of the sociology of education at the Catholic University of Santiago. Rada had played an active part in the running of the university as a student representative on the higher council. He was a known supporter of Allende's social change programme, and within days of the junta's takeover he stood up and spoke against the military rulers during a meeting of the higher council. He received a phone call during the night (from one of the regime's supporters as it happened) warning him that the junta had decided he was dangerous.

Rada sought refuge in a foreign embassy, and since he was unable to obtain a safe conduct he left the country by a route which he prefers not to disclose—although he is prepared to describe a fast car ride to the airport with motor-cycle outriders. He arrived in Britain earlier this year, and since he had not actually sat his final exams at the time of the coup he asked permission to qualify at London University. When the university wrote to Chile in order to learn about Rada's academic record there they were told that nobody of that name had ever been at the Catholic University of Santiago. As far as the university where Rada had served on the higher council was concerned, he had never existed. Rada was able to establish his bona fides, and to take his exam, and he is now a post-graduate member of London University's Department of the Sociology of Education. He is in touch, regularly, with former colleagues who remained in the country. Their reports, he says, add up to a single message: "Intellectual life is dead."

Business report

John Gribbin and Roger Woodham

Gordon and Breach Science Publishers Inc., of New York, have filed a petition under Chapter XI of the United States Bankruptcy Act. The company's 1973-74 list contains 85 journal titles, most of them scientific; but, according to the management of the British subsidiary of the company, production has not been affected by the filing.

The G & B petition itself follows a similar petition filed under Chapter XI of the Bankruptcy Act by a subsidiary, Media Directions Inc., on November 2, 1973. According to a representative of the British company, the petition now filed by the American parent on March 29, 1974, was necessary "to protect the parent from repercussions from that action".

The Managing Director of the British-based operation — a wholly owned subsidiary of the American parent says that "production, promotion, distribution and other publishing operations are not affected by the filing, and subsidiaries of the American company are not involved. I should stress that we intend to continue our normal publication of journals and new books."

The first meeting of the G & B creditors was held in New York on April 24, before Bankruptcy Judge Edward J. Ryan. The proceedings were adjourned until May 13. Under American law it is possible for a company to bring the court and creditors into the running of that company without ceasing to trade.

According to figures circulated to major creditors by attorneys for the \$4,161,297 and 21 cents, while assets total \$2,302,940 and 84 cents including an item of \$2 million for "stock in trade".

● "The most significant new product range in the history of the company" was how Mr Ralph Price, chairman of Honeywell in the United Kingdom, described the new Series 60 computers when they were launched last week. Only time will tell whether Honeywell has the upper hand when companies like ICL and IBM unveil their models for the late 1970s and early 1980s in the next year or two.

Honeywell's total turnover in 1973 was \$1,170 million, and the profit before tax was \$93 million. Although not actually disappointed with the growth of sales in the past year or two, senior management at Honeywell hope that sales will grow faster with the Series 60.

The advent of Series 60 is a positive move on Honeywell's part towards the marketing of fewer, but more flexible, pieces of hardware and software. The new range builds on the best of

Six weeks after the new Australian-Soviet science agreement was initialled in Canberra, it has been revealed that the Russian delegation which finalised these arrangements proposed at the same time that 'a joint scientific facility' be established in Australia. Although this was essentially a renewal of a previous proposal in 1971, the recent press stories caused a flurry of comment in the Parliament and diplomatic circles. There was reportedly a reaction from the United States Ambassador who was said to be concerned that the proposed 'facility' could be a threat to the various American defence bases in Australia.

Of these, two are in the big league—the naval communications base at North-West Cape (Western Australia) and the military satellite station at Pine Gap in the centre of the continent. A security screen surrounds the operations of these stations and there was immediate speculation that the Russians were 'trying out' Australia's diplomatic commitment to the American alliance by indirectly setting up a facility which could conceivably also act as an electronic monitor of American activities.

Russians seek joint facility in Australia

Peter Pockley, Sydney

Both the Prime Minister, Mr Gough Whitlam, and the Foreign Minister, Senator Don Willesee, were, however, at pains to emphasise that no decision had yet been made and that the proposed facility—to be managed jointly by both nations—would be 'to photograph space objects and to contribute to atmospheric studies'. It is not, as reported by some news agencies, intended to be a tracking station. The proposal is now being studied by various departments before Mr Whitlam's planned visit to Moscow in the middle of the year.

For readers in the Northern Hemisphere, who have become accustomed over decades to continual contact with the Soviet Union of a military and diplomatic kind, the sharp Australian reaction to such an apparently mild proposal may seem excessive. But, rare

though it is in the Southern Hemisphere, even remote possibilities of direct influence by the Soviet Union on events here have always been greeted by over-reaction.

The most celebrated example in recent times was the case of Vladimir Petrov, a Russian diplomat-cum-spy operating out of Canberra, whose defection in 1954 was turned to long lasting political advantage over his Labour opponents by the then Prime Minister, Mr Robert Menzies. The first Russian scare occurred, rather extraordinarily, in the Crimean War when the new settlers actually built harbour forts in a show of self-reliant bravado against the imaginary invaders from the steppes.

The present concern has been a two-day political wonder but it has its roots in the fears of a possible buildup of Soviet naval power in the Indian Ocean on Australia's undefended western seaboard of about 2,000 miles. As with the Australian-Soviet science agreement itself, this latest matter was treated by politicians and press alike as primarily a diplomatic affair, with science as the passenger in the vehicle.

Honeywell's present lines and those of the erstwhile computer arm of General Electric (USA) which Honeywell took over three-and-a-half years ago. At that time Honeywell and General Electric actively marketed 10 computer series, 20 processors and 12 software systems—now there are just four, eight and six. The largest of the new series, Level 66 is based on the Honeywell Series 6000 which has brought in about \$1,000 million since it was launched in 1971.

The development costs for the new series have hardly been trivial; most of the \$50 million earmarked for development by Honeywell in each of the past three years has gone on the Series 60, and much of the development work was done in Europe. (Level 66 computers are, indeed, to be manufactured at the Honeywell factory in Newhouse, Scotland.)

Honeywell says that the Series 60 will be suited to existing computer users and newcomers alike. Prices for Level 66 systems start at £450,000—of £10,000 a month on a rental basis—but Honeywell do have relatively small companies and organisations in mind for the less sophisticated Level 61 which costs £22,000+£400+a month). Honeywell is using the phrase 'attack machines' to describe the Level 61, for it sees many potential customers buying this as their first computer, possibly to do such mundane jobs as stock control.

Cash problems for public interest law

Colin Norman, Washington

SINCE they first rose to prominence in the late 1960s, public interest law firms have made an impressive contribution to the development of government policies in the United States, ranging from the regulation of food additives to the protection of the environment. Staffed by a handful of lawyers and supported chiefly by philanthropic organisations, they have provided the cutting edge of the environmentalist movement and in many cases have formed an effective counterforce to the big business lobby which traditionally dominates the Washington political scene.

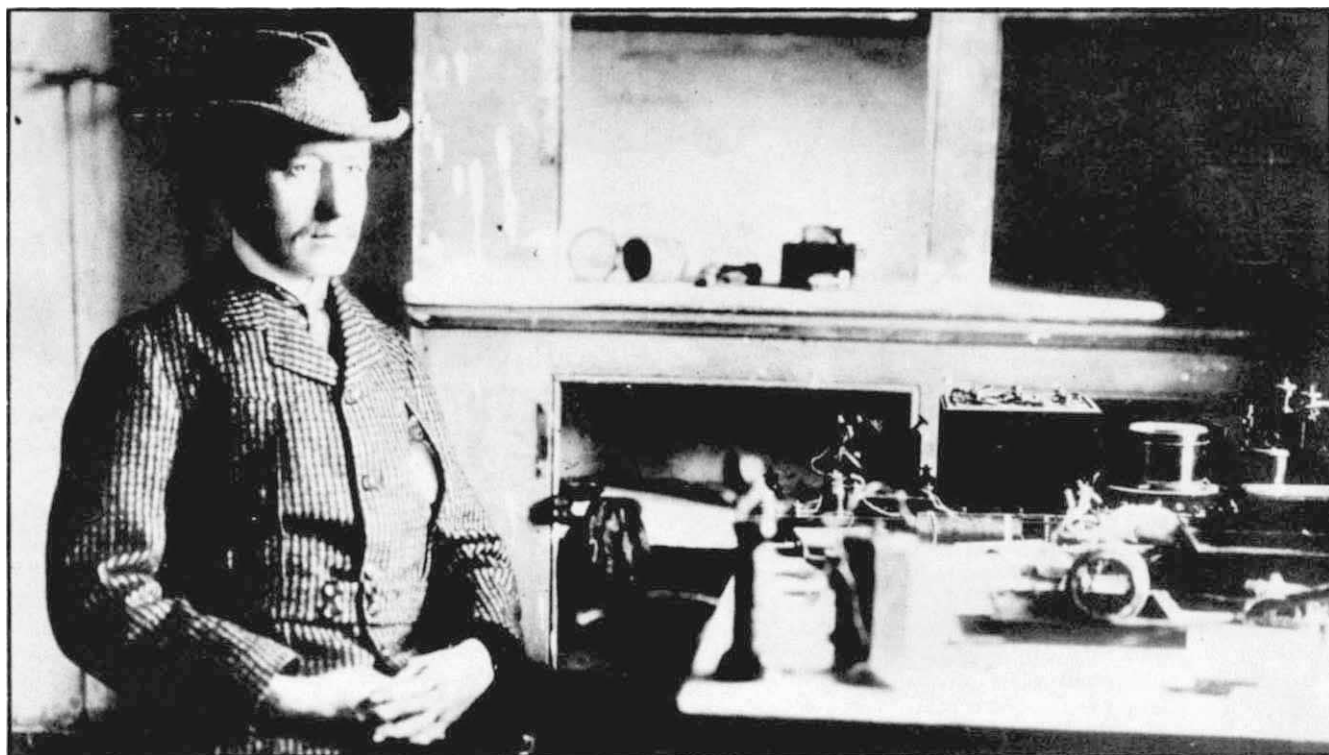
But they are now facing the prospect that one of their largest single sources of funding, the Ford Foundation, may withdraw its support in the next couple of years and put its money elsewhere. Ford has been pumping millions of dollars into public interest law since the 1960s, and since it traditionally provides seed money for the establishment of projects, rather than financial support over long periods of time its withdrawal from the field has long been anticipated.

Some idea of the influence of the foundation on the field of public interest law can be gauged from the fact

that it provides between 85 and 90% of the budget of the Center for Law and Social Policy, the law firm which was in the forefront of the legal battle against the trans-Alaska pipeline and which have been effective in setting standards for treatment of the mentally retarded. The foundation also contributes some 40% of the operating funds of the Natural Resources Defense Council, which has won several landmark court victories against the Environmental Protection Agency and which argued the law suit which eventually forced the Atomic Energy Commission to publish an environmental impact statement on the liquid-metal fast-breeder reactor programme.

Faced with such the prospect that their funds may dry up, many of the law firms are exploring possible new financial arrangements which will put their activities on a sounder footing and provide them with a more permanent source of funds.

Two recent events have brought the matter to a head. The first is that last week the Ford Foundation announced that it will provide four of the largest firms with \$2.3 million to finance their activities at least until the end of 1975, but it accompanied the announcement with the statement that Ford's role after the grants expire "cannot be determined now". And the second is that the Ford Foundation itself convened a conference in San Diego in



Marconi at Signal Hill, Newfoundland, in 1901 with instruments used to receive the first trans-Atlantic wireless signal.

THE Science Museum, London, is commemorating the centenary of Marconi's birth with an exhibition which opened on April 25. This is more likely to appeal to someone with a keen interest in wireless or in the history of science than to the casual visitor; the most interesting exhibits are letters and other literary material which are clearly presented and easy to read, but require time to digest.

Other exhibits emphasise the social rather than the scientific significance of wireless, with pieces of original equipment (including the kite used to fly the aerial from which the historic transatlantic broadcast of December 12, 1901, was made) and personalia including Marconi's swordstick.

One of the curiosities on show is a copper earthing tube dug up in the 1960s from the garden of the bungalow where Marconi lodged in 1896-97; the tube was in the ground outside Marconi's old room and was probably used by him in his earliest experiments in Britain.

But the written and printed matter is by far the most interesting. A cutting from the *New York Times* of February 13, 1927, reports Marconi's views about the prospects for wireless transmission of power, "radio movies" and television. He was rather optimistic about the latter, and the reporter looks forward to the day when "Secretary Kellogg (sits) in his office in the State Department and converses face to face

with Premier Baldwin in Downing Street". In other ways, Marconi was equally visionary, foreseeing the replacement of oil as a fuel source by solar energy, but dismissing the idea of tapping geothermal power because heat from the Sun is both a simpler and cheaper source.

By 1932 (according to the *Daily Mail* of December 6) Marconi's vision had expanded still further. Musing that messages could be sent on further than around the Earth, and prompted by the interviewer, he said: "Interplanetary communication—who can tell? There is a great deal more to be found out and made perfect."

John Gribbin

mid-April to discuss possible new arrangements for financing the firms.

Although the foundation has made it clear that the firms should be thinking seriously about how they should live without Ford money, it is equally clear that if it came to the crunch, Ford would be unlikely to pull the rug from underneath public interest law firms and allow them to expire or cut back their operations drastically. Thus the emphasis will be on finding new sources and methods of finance.

Two possible arrangements were suggested in the San Diego meeting and, although no concrete decisions were taken, at least two possibilities were given careful consideration and hold

great promise. One idea is to set up a permanent fund, financed by private donations, philanthropic organisations and perhaps even by professional legal organisations. Such a fund would probably operate much like a foundation, but the way in which the money would be distributed has not yet been worked out.

Another possibility is that courts will award legal fees to public interest law groups which successfully sue the government and industry. But the problem there is that the acceptance of such fees could well endanger the firms' legal status as tax-exempt organisations, and until the Internal Revenue Service issues some guidelines on the

matter the firms are precluded from taking such fees. Ironically, some major pieces of environmental legislation, such as the Clean Air Act, and the Federal Water Pollution Control Act contain specific provisions which direct courts to award attorneys' fees to organisations which successfully bring court cases against the government, but until the IRS gets round to issuing its guidelines, those fees cannot be awarded to public interest groups.

Thus the next couple of years could be critical for the future of an extremely important element in governmental decision making in areas which have a profound effect on scientific matters.

IN THE late 1960s travellers' tales suggested that Russians were having some success in detecting premonitory symptoms to earthquakes. Physical properties of rocks in Central Asia were said to change prior to quakes, although Western geophysicists, deeply immersed in plate tectonics and lacking any sight of the Russian data, were not moved to do much. Then in 1972 American interest suddenly grew explosively. By early 1973 precursors to earthquakes had been seen in many parts of the world, a dilatancy theory had been proposed to explain the observations and a simple relationship between the duration of the precursor and the size of the earthquake had been suggested. The annual American Geophysical Union meeting in April 1973 was a heady experience. This year's meeting was sober by comparison, and there was much concern about funding.

The situation is a classical one of what one could call the four black spots of present day science policy:

- Too many people chasing too little money.

- Government, industry and universities working in the same field.

- The political need for a mission-oriented approach in conflict with scientists' desire for in-depth understanding.

- The problem that the contractual system poses for the university scientist.

On the scientific side, it had been a relatively quiet year. There had been two instances, one in New York State, the other in Riverside, California, where events had been foretold by means of variations in seismic wave travel times before the earthquake. The same technique was also showing some promise in other places, although there seemed a growing feeling that the quality of data was often inadequate and that the near-focal conditions are vastly more complex than a first modelling would suggest. On the other hand there is promise both in gravimetric and tilt observations and some of the laboratory and field experiments (including earthquake control by fluid injection) have gone well.

Responsibility for the American 'earthquake hazard reduction programme' is vested in the US Geological Survey (USGS). Much more than prediction is covered—risk analysis, mapping and rock mechanics are other fields of activity. In the fiscal year July 1971–June 1972 (FY 1972) the programme amounted to \$1.6 million, but the impact of the San Fernando earthquake put up the allocation to \$4 million by FY73. In moving into FY74, the first year in which prediction could be discussed seriously, the USGS found itself picking up responsibility for earthquake work previously done by the National Oceanographic and Atmos-

Quake prediction no bonanza

David Davies

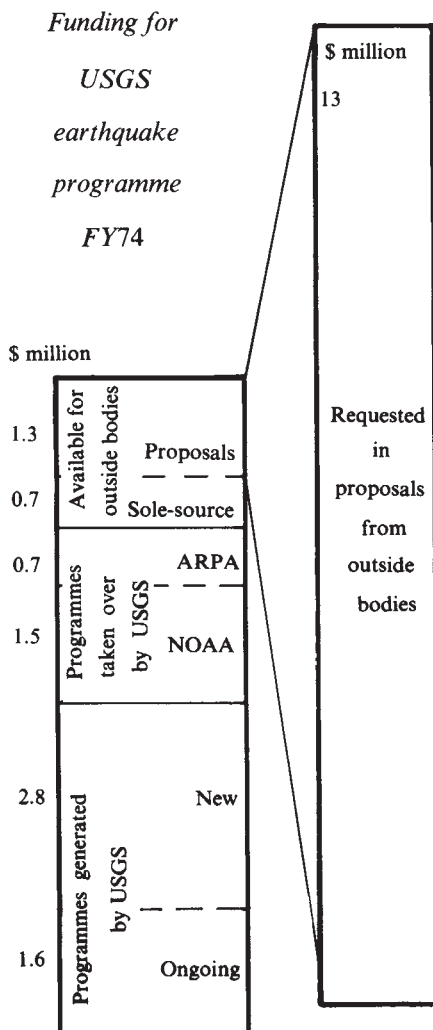
pheric Administration (NOAA) and fluid injection studies of the Advanced Research Projects Agency (ARPA). Thus although the budget for FY74 was \$8.6 million, by no means all the increase was in the form of new money for research. The figure shows the distribution. Apart from sole-source contracts—for instance to support seismic networks operating at present, universities, state governments and industry had \$1.3 million to fight over. No increase is seen in this sum for FY75. "We need a big earthquake to shake more money out of the government" as one official put it.

The figure of \$1.3 million took many by surprise, as it was generally expected that in the mid-1970s the USGS would dispense the largesse that in the past had come from ONR, NASA, NSF and ARPA and which had made for so many thriving geophysical laboratories. External funding applications for FY74 amounted to \$13 million from two

hundred proposals—the USGS is able to support 32 of them. On one official's reckoning it could have intelligently used \$8 million this fiscal year. Expertise is going unused, he thinks. As it is, some seismic stations in California have already closed for lack of funding. Other agencies no longer have a charter to do research in this field, so failure with the USGS really does mean failure to fund. The prediction programme is a very lean operation in which immediate relevance is necessary of every project. "A hard-nosed mission-oriented approach" as one project officer described it—himself with a modest fame as having already engendered such an approach in three other government agencies.

It is inevitable that there is conflict in such an underfunded situation. More generalised and global research suffers at the hands of the more particular and American. There is a suspicion that the USGS will swallow up all the good ideas that come from outside (university scientists pointedly remark that the first good papers in the subject came from MIT, Stanford, Columbia and Caltech, not the big government laboratories which now have most of the available money). And, most immediate, people in universities suddenly find themselves up against government contracts officers, zealous to ensure scrupulous accounting. It is this more than anything else which seems to have persuaded rational men that they are being persecuted. The staff of the USGS are aware of these undercurrents of bitterness and are trying to establish better headquarters procedures.

There are some very obvious defects at the moment and action needs to be taken at governmental level (university scientists are also going to have to learn to live with the system a bit more). The lack of funds is quite ludicrous. The United States loses on average \$630 million annually through earthquake damage—a figure which will doubtless go up when California next suffers a serious quake. To keep talent out through lack of one-hundredth of that sum is a false economy. Worse, it is worrying that the programme is required to be so nationally oriented (the USGS is, of course, part of the Department of the Interior). Most countries look to their own scientists to solve their own problems, but the Western world has increasingly looked to the United States to grapple with global problems, at risk of being branded imperialists. If ever there were a problem knowing no frontiers this is it, and success would be that much more likely if more American geophysicists could be financially encouraged to think and collect data in a more global framework.



correspondence

Russians at conferences

SIR,—“Have the Russians arrived?” International conference-goers will know how the question passes from one to another as they assemble at the start of the meeting. The uncertainty of whether the Soviet delegation will appear or not (and if they appear, on which day?) has long provided a touch of drama for the other participants and a headache for the organisers. The first clue for the organisers is often a message from the travel agency acting for Intourist that they are asked to book hotel accommodation for so many Soviet delegates. The names may not be known, only the total number, and the scientific contact with them will commonly not be direct but through some official Soviet committee or authority.

I have recently returned from an international conference abroad where this scenario was duly re-enacted, but this time I experienced it directly as I had to preside over the meeting. Writing now in my private capacity, not as president, I have to report sadly that nothing has changed; it is as bad as ever.

We had asked a prominent Soviet scientist, let us call him Professor Y, to give one of the specially invited review papers and our invitation was accepted. As the time drew near the secretary of the meeting wrote to him several times to ask about his paper, but with no reply. Telegrams were tried, and then many telephone calls to Moscow. Professor Y was unknown to them, they said; or, Professor Y had gone away. Faced with this concerted silence, we began to fear for our colleague. Finally, four days before the opening of the conference we received a telegram from Professor Y that his paper would be presented on arrival. (All the other papers for the conference had already been reproduced for distribution.) Our hopes rose, but we remembered the old Russian proverb “Do not sell the bear-skin until you have shot the bear.” The Soviet delegation, expected to be twelve, turned out to number seventeen, including the usual chaperone from Intourist, and Professor Y was not among them. They bore with them copies of his paper for distribution and a verbal message from him asking me to present it. I did so, believing that my colleague was in no way to blame for these bizarre events and wishing to help him in his difficul-

ties as much as I could (the rules of the conference just allowed it). It is worth adding that some or all of the five extras in the Soviet party did not have enough foreign currency to pay the fee and so were unable to be registered for the conference.

The Soviet authorities who are responsible for these things should realise that this sort of behaviour on their part is harmful to Soviet science. Organisers of conferences already think twice before inviting papers from Soviet authors, knowing the uncertainties that will result. We should like to include them, on equal terms with the others, but how can we go on doing so in face of this continued official discourtesy to the scientific community?

Yours faithfully,

J. D. NYE

H. H. Wills Physics Laboratory,
University of Bristol, UK

Great crested grebes



The Zoological Society of London

Darwin's omission

SIR,—I wish to draw the attention of historians of science to the omission by Darwin (in his *Origin of Species* and even in his *Selection in Relation to Sex*) of any mention of cases where the two sexes are similar in plumage. Sexual selection for Darwin involved the choice by females of brighter, and often larger, males. Darwin presumably did not have the opportunity to study sexually similar species: once he returned to Down House he was not able to observe freshwater or marine birds.

The same omission occurs in the books on evolution by Ernst Mayr, in Bernard Rensch's *Evolution above the Species Level* and, strangely enough, in the early edition of my own *Evolution, the Modern Synthesis*. I say strangely,

for in the years 1914 to 1917, I myself had described the elaborate mutual display of grebes, herons and egrets; and later returned to the display of swans, albatrosses, and penguins, which had been studied in detail by others. The first account of mutual display was given by E. Selous in 1901–02, and by myself in 1914; in both cases, of great crested grebes (*Podiceps cristatus*, *Proc. zool. Soc. Lond.*, 1914). My failure to mention mutual display in my book seems to have been due to my not realising its frequency or its biological value in strengthening the emotional bond between members of a pair. This point emerges clearly from the work of Lorenz and Tinbergen.

I wish to stress the strikingly different results of unilateral and mutual display, in regard both to display characters and to behaviour. A regular feature of mutual display is that the returning bird presents the sitting mate with nest material (in grebes a similar presentation occurs on open water). Another feature of mutual courtship is the extent of ritualisation; but this also applies to many male unilateral displays where the sexes have different plumage, as in bustards, pheasants, some grouse, and so on. Today the importance of ritualisation has been confirmed: see the Royal Society's symposium on Animals and Man (*Phil. Trans. R. Soc.*, 1966).

The red breast in both sexes of the robin (*Erithacus rubecula*) is not connected with courtship: Lack has shown that the colour is a mark of territorial ownership, for both sexes have winter territories. In dimorphic species the songs of males, and of 'duetting' birds such as some African Shrike, are also proclamations of territory.

Yours faithfully,

JULIAN HUXLEY

31 Pond Street,
London NW3, UK

Rat race

SIR,—a recent advertisement emphasises the present crisis in biology laboratories, where research is severely hampered by the shortage of experimental animals. “A position of Wissenschaftlicher Rat is vacant” in Freiburg (*Nature*, March 1, xx; 1974); applicants should have experience of cell-mediated immune reactions.

Yours faithfully,

B. JOVE

Hove, UK

news and views

Transferring foreign genes to plants

It is well established that purified DNA can be taken up by bacteria and that the recipient cells are capable of incorporating DNA into their chromosomes and expressing the genes thus acquired. Attempts to perform similar experiments with eukaryotes, however, are often viewed with scepticism although an increasing number of workers are investigating this problem.

In their simplest form these experiments involve the use of two closely related organisms which differ with respect to an easily identifiable phenotypic characteristic. Attempts are then made to transfer this character to individuals that lack it by applying purified DNA from the appropriate donor. The experiments of Hess involving white and red flowered plants of *Petunia* fall into this category (Hess, *Z. Pflanzenphysiol.*, **60**, 348-358; 1969; **61**, 286-298; 1969; **63**, 461-467; 1970; **68**, 432-440; 1973). In some instances the test organism has a deficiency in an essential metabolic pathway and corrected individuals are selected by their ability to grow without the appropriate nutritional supplement. In many experiments the donor DNA is derived from organisms totally unrelated to the recipient and evidence for uptake and possible incorporation into host chromosomes has been obtained. Occasionally bacteriophages have been used as an enriched source of specific genes and there is evidence for the synthesis of enzymes coded for by the phage DNA (Carlson, *Proc. natn. Acad. Sci. U.S.A.*, **70**, 598-602; 1973).

To establish that true transformation is possible in eukaryotes, it is necessary to study the fate of applied DNA by biochemical techniques, to show that changes in the recipient organism are directed by the donor DNA and to demonstrate that these changes are inherited. Ledoux and his colleagues have been carrying out some of these experiments for more than a decade and have obtained some remarkable results (Ledoux and Huart, *Eur. J. Biochem.*, **23**, 96-108; 1971). In this issue they present data which suggest that thiamine deficiency in *Arabidopsis thaliana* may be corrected by bacterial DNA and furthermore that this correction can be inherited.

Arabidopsis is particularly suitable for such experiments. It is comparatively small and easy to manipulate and grow aseptically and it flowers within a few weeks of germination. Ledoux and his team used a number of lines with lesions in the branched pathway for thiamine biosynthesis. These plants normally require either exogenous thiamine, pyrimidine or thiazole for continued growth and reproduction but they found that a small number of the plants grew and produced seed in the absence of supplements after they had been treated with the appropriate bacterial DNA.

Ledoux states that DNA with a molecular weight in excess of 10^7 is necessary and the DNA solution should be applied to the ungerminated seeds during imbibition. DNA-corrected plants grow more slowly than supplemented mutants and only about one third of those that grow actually produce seed. The observed frequency of correction can be as high as 10^{-2} compared with a spontaneous reversion rate of the *Py* locus of less than 5×10^{-6} , and 4×10^{-5} for reversion induced by ethyl methane sulphonate. DNA from phage T7 and 2C is ineffective and DNA from *Escherichia coli*

unable to produce the thiazole moiety of thiamine does not correct the corresponding *Arabidopsis* mutant although it is effective with plants deficient in the pyrimidine pathway.

Seeds from corrected plants were germinated and selfed to produce F_1 to F_3 progeny. Although these plants showed some variegation in chlorophyll content no typical thiamine-deficient segregants were observed. This suggests that the parental lines are homozygous which is a rather surprising result although it should be noted that Hess observed a similar situation with *Petunia*. Ledoux suggests that in the case of *Arabidopsis*, during meiosis and afterwards, the gametes containing the information for thiamine production are favoured over mutant gametes. In support of this proposal he cites the finding that if during DNA correction plants showing signs of abortive flowering are supplemented with thiamine then lethal mutants appear in the F_1 . Although this result would also be expected in the absence of applied DNA, in this case no corrected plants would be anticipated. In crosses between progeny of DNA-corrected plants and either wild type or mutant individuals, lethal mutants and a variety of leaky mutants are obtained. There is no evidence for cytoplasmic inheritance of the corrected character.

Obviously the mechanism involved in these DNA-mediated corrections is of major interest, but it would be premature to attempt a detailed interpretation. From the figures quoted by Ledoux the frequency with which the corrections are obtained is too high to be explained by spontaneous reversion of the mutation. On the other hand direct evidence for the synthesis of a bacterial enzyme in these plants is not available at present. Other experiments by the Mol group suggest that foreign DNA can become integrated in some way into plant DNA although the significance of these findings has been questioned (Hotta and Stern, in *Informative Molecules in Biological Systems* (edit. by Ledoux) 176-178, North Holland; 1972). One thing is certain; experiments of this nature hold out intriguing possibilities and they will continue to generate interest and occasionally heated discussion.

From our Plant Cell Physiology Correspondent

Number of cells at the time of X activation

ALTHOUGH female mammals possess two X chromosomes, in any particular adult cell only one of these homologues is active, providing a dosage compensation which ensures that females and males both display the same number of active-X-linked genes. The fertilised egg and very early embryo of eutherian mammals, including man and mouse, possess two active X chromosomes; that one of these two chromosomes is randomly inactivated in each cell at an early stage of genetic development was first suggested by Lyon (*Nature*, **190**, 372; 1961).

The random nature of the process is suggested by the expression of sex linked genes in adult females. In heterozygotes in which one X chromosome carries a wild type and the other carries a mutant allele, those cells in which the wild type X chromosome is active must show wild phenotype whereas cells instead possessing the active mutant X chromosome display the mutant phenotype. One visible

consequence of this random inactivation is the variegation seen in the coats of female mice heterozygous for X-linked coat colour genes. The equal representation of wild and mutant phenotypes, and the presence of patches of each coat colour, suggest that at the time of decision in the early embryo, in each cell one X chromosome (chosen at random) remains active whereas any others are inactivated (in animals possessing several X chromosomes only one stays active). In the subsequent divisions of genetic development, the state of each homologue is faithfully reproduced; the adult cells descended from any particular embryonic cell after the time of decision therefore constitute a clone characterised by the presence of the same active X chromosome (with the exception of the germ cells in which both X chromosomes remain active).

Two critical questions to ask about genetic development in eutherians are therefore: when does X activation take place; and what is the basis for its random nature? The closest estimate of the time of decision derives from the work of Gardner and Lyon (*Nature*, **231**, 283; 1971) with chimaeric mice. Instead of the usual complete fusion between two embryos, they injected a cell taken from a blastocyst into another blastocyst. Because the donor and recipient carried different gene markers for coat colour, any cells in the adult coat descended from the injected embryonic cell can be distinguished. Since donor sex-linked markers showed

variegation in the heterozygous condition, X activation must take place after the blastocyst stage (otherwise the donor cell would have suffered previous X activation and its descendants would all be of one phenotype in the adult).

By taking advantage of a mutant which influences the random nature of X activation, Ohno and his colleagues (*Cell*, **1**, 175; 1974) have recently developed an ingenious approach to estimate the number of cells in the embryonic mouse at the time of decision. Their experiments rely on the concept that the clonal composition of embryos immediately after X activation can be expressed as the binomial $(p+q)^n$, where n is the number of cells and p and q are the probabilities of activation of each chromosome, both equal to 0.5 in the wild type animal. In other words, given a population of animals it is possible to predict the frequencies with which any particular clonal composition will be generated. Unfortunately, this formula cannot be directly extrapolated to the adult animal, because distortions in the initial clonal composition must inevitably take place as the embryo grows; for only some, and not necessarily a random selection, of the embryonic cells will be utilised during formation of each adult clone. The formula which determines the clonal composition of an adult phenotype must therefore be written as $(p'+q')^{n'}$, where n' is the number of progenitor cells from which the adult clone is descended, and p' and q' have values corresponding to the relative frequencies of each active X chromosome in the set of progenitor cells—and this, of course, in turn depends upon the clonal composition of the embryo from which the progenitor cells are descended.

Because these changes in cell composition make it impossible to determine n directly from the adult phenotypes, Ohno *et al.* have used a special situation to allow this analysis. In one particular condition only, changes in cell composition cannot occur during development; this is the case when all the copies of one X chromosome (either the maternal or paternal) were by chance activated. This generates a monoclonal embryo, which can in turn give rise only to monoclonal adult. But these two extreme cases each occur with a very low frequency (0.5ⁿ); as Ohno *et al.* point out, this creates the paradox that, even given a value for n as low as 20, such fractions would occur with frequencies too low to determine by experiment in any researcher's lifetime (of the order of one in a million mice). To achieve a more manageable situation, they therefore utilised a mutation of the X chromosome (0^{hv}), which they have previously characterised, and which causes preferential activation of the X chromosome carrying it in heterozygotes where the other chromosome is wild type (*Nature new Biol.*, **245**, 92; 1973; *Cell*, **1**, 3; 1974). The 0^{hv} mutation seems to be an extreme version of alleles at the Cattanach's "controlling element" locus which maps in the middle of the mouse X-linkage group; and earlier analyses of chromosomes carrying this allele have suggested that the mechanism of decision is for one X chromosome to be activated whereas any others in the cell are inactivated (reviewed by Brown and Chandra, *Proc. natn. Acad. Sci. U.S.A.*, **70**, 195; 1973).

When the 0^{hv} X chromosome carries the mutant gene *Blo* and the other X chromosome carries the corresponding wild type allele, regions of the coat of the mice are light coloured if derived from cells in which the 0^{hv} *Blo* X chromosome was activated, and are dark if derived from cells in which the wild type X chromosome was activated. Because of the preferential activation of the 0^{hv} X chromosome, the frequency of occurrence of mice with coats largely of *Blo* phenotype is appreciable. To obtain values for the occurrence of two, rather than just one, of the possible clonal compositions, in addition to assessing the proportion of mice with coats derived entirely from *Blo* cells, Ohno *et al.* also determine the frequency with which mice displayed coats almost entirely of *Blo* phenotype, but with just one dark spot (corresponding to one coat progenitor cell in which the wild

BL Lac identified with giant galaxy

THE peculiar object BL Lacertae seems to lie at the centre of a normal giant elliptical galaxy. This identification provides an estimate of the distance to BL Lac, and this in turn suggests that the central source is similar in spectral index and luminosity to QSOs such as 3C48, 3C279 and 3C345, provided these objects are at the distances indicated by a cosmological interpretation of their redshifts.

Those are the principal conclusions reached by Oke and Gunn as a result of a four year study of BL Lac (*Astrophys. J. Lett.*, **189**, L5; 1974). The observations were made with the multichannel spectrometer attached to the 200-inch telescope; some of the observations were made using a disk with diameter 5" and a surrounding ring with inner diameter 10" to study the extended regions of the source while obscuring light from the bright central feature, and these showed H β in absorption at a redshift of $z = 0.07$.

Other data support this redshift estimate, and show that the galaxy is one of the most luminous known, with a magnitude of about -23. Together with the growing evidence of similarities between BL Lac and QSOs this identification is one of the key advances of recent years. Far from being a blueshifted QSO, as had seemed possible, BL Lac can now be fitted into the cosmological picture of receding QSOs quite satisfactorily. The distance to the source is some 350 Mpc, and this raises the same questions about its energy generation mechanisms that apply for very remote QSOs. The energy requirements are extreme, but as Oke and Gunn say "It is interesting, however, that the same phenomena at approximately the same level of outrageousness exist for BL Lac as for higher-redshift QSOs if they are placed at the distances suggested by their redshifts".

J. G.

type X chromosome was activated).

These frequencies should have been determined by the clonal compositions of the cells which provide the immediate progenitors for the coat, that is they depend on the formula $(p' + q')^{n'}$, and so provide experimental values for the frequencies of the fractions $(p')^{n'}$ and $(p')^{n'-1}.q'$. To relate these values to the initial clonal compositions at the time of X activation (that is to p , q and n) requires knowledge of n' . Given a value for n' , it is then possible to test various combinations of p , q and n to see which values would generate cell populations from which animals monoclonal for the coat would be produced at the observed frequency. This involves summing several possible situations, since, for example, a monoclonal coat may arise not only whenever the original embryo was monoclonal but also whenever the progenitor cells of the coat by chance were descended only from cells with an active O^{hv} X chromosome; when the number of immediate progenitor cells is sufficiently large, the entirely mutant adult phenotype should occur only in individuals which started their development with very one sided clonal compositions.

The *Blo* mutation is therefore particularly well suited to such analysis, for the development of the cells in which it is expressed has previously been studied in some detail. *Blo* acts in mesodermal cells of somite origin, thought to result from the reproduction of 35 pairs of somite clones distributed along both sides of the vertebral column (Lyon, *Phil. Trans. Roy. Soc.*, **B259**, 41; 1970), suggesting a value for n' of 70. Animals with coats entirely of the *Blo* phenotype are too rare to measure in appreciable frequency, so Ohno *et al.* measured the frequency of occurrence of animals with a coat one half of which (corresponding to one side of the vertebral column) shows the *Blo* phenotype. This frequency, 41 in 2400 or 1.7×10^{-2} , provides an estimate of the frequency of the class p'^{35} ; the square of this frequency, 2.9×10^{-4} , can thus be taken as an estimate for p'^{70} .

This total frequency might be produced by several combinations of p , q , n . To decide upon the most likely, Ohno *et al.* utilised their estimate of the frequency of occurrence of the class $p'^{69}q'$, that is the proportion of animals with only one wild type clone apparent in the coat; this was 3 in 2,400 or 1.25×10^{-3} . Although the accuracy of this estimate is not great, because of the small number of animals involved, this value can usefully be compared with the estimate for p'^{70} . Taking both values into account, Ohno *et al.* were able to show that a close fit to the data would be produced by $p=0.8$, $n=60$. In other words, the O^{hv} allele confers an 80% probability that its chromosome will be

activated; and the number of cells in the embryo at the time of decision may be close to 60.

How accurate is this estimate and what further tests may be made of it? One assumption upon which these experiments rely is, of course, that all 35 pairs of somite cells arise independently from some undifferentiated pool and not from a smaller pool of cells which then multiply upto 70. It would be useful if this point could be confirmed in future. Given the comparatively small numbers of animals which can be examined under the experimental conditions imposed by this system, the measured frequencies must clearly be taken as approximations and not precise values; but the value for n of 60 cells is consistent with the previous suggestion that the time of decision is soon after the blastocyst stage. It is therefore likely that O^{hv} influences only the decision on activation and does not change the time at which it is made. Increased accuracy for these estimates would obviously be lent by determining clonal compositions and thus values for p , q and n from some other adult phenotype. But these qualifications do not detract from the importance of this seminal study, whose sophisticated manipulation of the mouse system has provided a useful estimate for the cell number in a mammal when the decision is taken on X activation.

B.L.

More about Mercury

by our Cosmology Correspondent

MARINER 10 has done more than just send back some impressive pictures of Mercury, looking so very much like the Moon that one radio astronomer remarked recently that for all he knew they were in fact previously unreleased Lunar Orbiter photographs. The six non-imaging experiments on board the spacecraft have provided a new insight into the nature of the closest planet to the Sun, and this shows clearly how Mercury fits into the overall picture of the Solar System.

The photographs have, of course, contributed to this. The front cover illustration this week shows a region very similar to the terrain of the Moon, with a large, flat bottomed crater about 100 km in diameter (about the same size as the lunar crater Copernicus), linear grooves probably produced by crater ejecta, and so on. Together with pictures of Mars obtained by the Mariner series of spacecraft and by Soviet spacecraft, the latest NASA achievement emphasises the broad similarities between the inner planets of the Solar System. It now seems almost beyond doubt that Venus too will be found to be cra-

Littorinid group

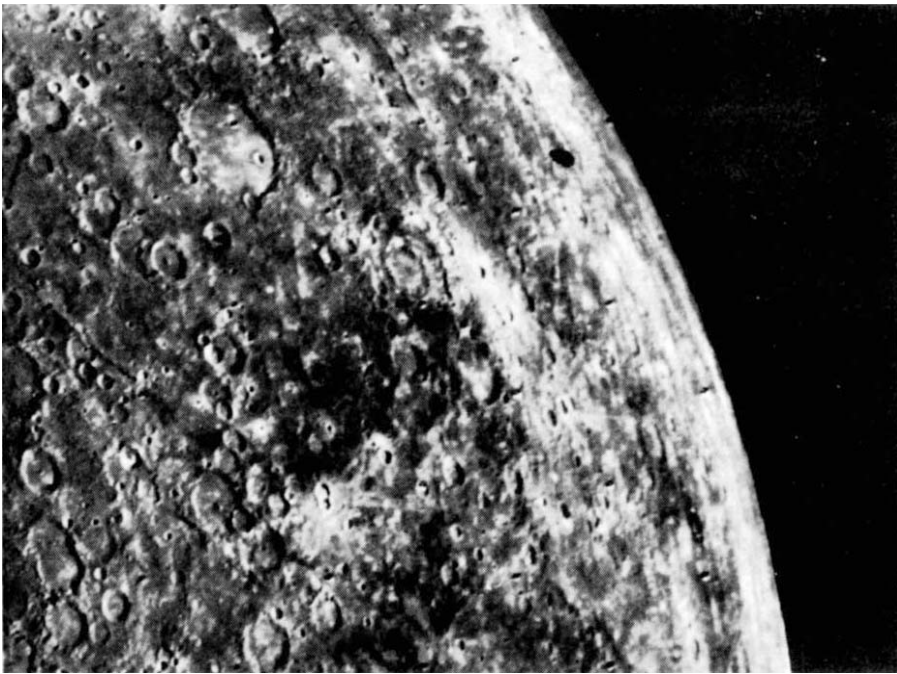
LITTORINIDS are present on the rock shores of all continents and present some intriguing taxonomic problems. The first symposium restricted to this family of the prosobranch Gastropoda was held at the Royal Scottish Museum, Edinburgh on March 19 under the chairmanship of Sir Maurice Yonge. Of the talks on taxonomic trends in the Littorinidae, perhaps the most noteworthy contribution was the evidence of J. Heller (University of Liverpool) that the polymorphic species *Littorina saxatilis* (Olivi) should be regarded as containing at least four separate species.

The meeting unanimously endorsed the suggestion that an informal littorinid group should be formed, with an international membership, as far as possible comprising all workers actively interested in these gastropods. Anybody wishing to learn more about the group should write to the appointed coordinator C. Pettit, Manchester Museum, The University, Manchester M13 9PL, England.

tered and scarred by impacts and volcanic activity, although the evidence there may have been largely eroded away.

If the overwhelming impression of the visual evidence is that the inner Solar System forms a family of related planets, the non-visual data confirm this and indicate the way in which the distance of each planet from the Sun determines its individual characteristics within the family. Mercury turns out to be rather more massive than was expected, with a density of 8 g cm^{-3} implying that as much as two-thirds of it is composed of iron or iron group elements. The presence of a clearcut gradient in the densities of the planets in sequence outwards from the Sun obviously ties in neatly with the idea that more volatile elements condensed further out in the collapsing proto Solar System. And a large amount of iron could also explain the unexpectedly large magnetic field of Mercury. At some 200 to 300 gammas for the extrapolated strength of the field at the planet's surface, this is about 1% of the Earth's field; the favoured view at present is that this is a permanent field, and is not induced by a dynamo effect.

Among the other surprises already arousing interest is the discovery of a tenuous atmosphere around Mercury. This discovery will delight amateur astronomers, since reports that such an



Southwestern quadrant of Mercury, viewed from Mariner 10 4 h before closest approach. Distance of spacecraft from planet was 198,000 km; the largest craters visible are some 100 km across. (NASA photograph.)

atmosphere could be seen have been made by members of that fraternity from time to time, but met with a cool reception from the professionals. Wise after the event, those professionals now say that such a tenuous atmosphere can be maintained, in spite of the low escape velocity from the planet, because of particles released through radioactive decay. Uranium releases helium (alpha particles), and potassium yields argon; another effect, capture from the solar wind, probably accounts for the presence of neon.

Clearly it will take a while for the new information to be digested and incorporated into theoretical models, and in September there is the prospect of more information as Mariner 10 passes by Mercury again. In the meantime, it is interesting to reflect on a comment attributed to Bruce Murray, leader of the Mariner 10 TV experiment team (*Aviat. Week Space Technol.*, **100** (14), 16; 1974), which indicates the rapid development of spacecraft systems and also answers the 'doubts' of that radio astronomer. The Mariner 10 pictures cannot be directly compared with Lunar Orbiter pictures, it seems, "because the Mariner 10 photos show more detail".

Models of growth and competition

from our *Animal Ecology Correspondent*

THE dynamics of competitive interaction between two species have long been the target of approximations. The

formula

$$\frac{dN_i}{dt} = r_i N_i (1 - N_i/K_i - \alpha_{ij} N_j / K_i)$$

(where N_i is the density of species i ; r_i is the exponential growth rate of this species when its population density and that of its competitor j are low; K_i is the carrying capacity of the environment for species i when its competitor j is absent; α_{ij} is the linear reduction (of K_i) of species i 's growth rate caused by its competitor j) has stood for more than 50 years since being proposed by Lotka (*Elements of Physical Biology*, Williams and Wilkins, Baltimore, 1924) and Volterra (*Leçons sur la Théorie Mathématique de la Lutte pour la Vie*, Gauthier-Villars, Paris, 1931). The model was validated in 1934 by Gause (*The Struggle for Existence*, Williams and Wilkins, Baltimore) who studied the dynamics of yeast and protozoan competition. The linearity of the model suited this kind of interaction. For arthropod interactions the model is less perfect. Gilpin and Ayala (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 3590; 1973) have recently put the model to the test using arthropod species and have come up with some important modifications.

Their competing species were *Drosophila willistoni* and *D. pseudoobscura*. Adult flies, in known densities, were put in culture vessels containing known amounts of food. After 1 week the surviving adults were counted, as were their offspring which emerged from the culture medium during the following 4 weeks. Zero isoclines, which depict the separation point between positive and

negative population growth, were constructed from continuous populations, maintained by serial transfer. The single species equilibrium, or environmental carrying capacity, is the maximum population the culture medium can stand. As a competitor increases in abundance the zero isocline falls away from the maximum point. Where it intersects the zero isocline for the competitor species is the equilibrium point for the interaction of the two species.

The data from the *Drosophila* cultures were fitted to a large number of competition models chosen according to the criteria of simplicity, reality, generality and accuracy. The model that stood up best was

$$\frac{dN_i}{dt} = r_i N_i (1 - N_i/K_i)^{\theta_i} - \alpha_{ij} N_j / K_i$$

The interpretation of the parameters is straightforward. r_i is the exponential rate of growth of species i when its density and that of species j is low; K_i is the carrying capacity of the environment for species i in the absence of its competitor j ; α_{ij} is the reduction of growth rate of i as a result of the activities of j , and θ_i denotes the asymmetry of the single species growth rate of species i . In the absence of competition, that is when $\alpha=0$, the growth rate conforms to the logistic. In other words the relationship between growth rate and density is parabolic. The point of maximum growth rate is $K/2$. The experimental data pointed out this restriction. The addition of θ removes it and allows the point of maximum growth rate to be less than, or more than, $K/2$.

This is an important modification to the Lotka-Volterra equation. Vertebrate populations in which growth rate tends to be controlled abruptly by the imposition of territoriality and other social factors cannot adequately conform to the continuously linear fashion demanded by the old model (when $\theta=1$). For them θ will tend to be larger than 1. Invertebrate populations, in which eggs, larvae and pupae are ignored, will tend to have a θ value less than 1.

Gilpin and Ayala propose this new model to encompass intraspecific and interspecific competition. Population ecologists shall have to wait before assessing its general applicability.

Biogenesis of chloroplasts and mitochondria

from a Correspondent

RECENT work on the biogenesis of chloroplasts and mitochondria was discussed at a symposium of the British Society for Cell Biology at the University of Warwick on March 28-29. Both

types of organelle are potentially autonomous, since they each contain DNA, DNA polymerase, RNA polymerase, and a protein-synthesising system, but all the evidence suggests that most organelle proteins are encoded in the nucleus and are synthesised by cytoplasmic ribosomes. The major problems discussed were the identification of the genes in the organelle DNA and of the proteins synthesised by organelle ribosomes.

The interactions between nuclear and organelle genomes in the synthesis of organelle proteins are complex, but are yielding to modern techniques. Two general principles are emerging: (1) one of the functions of the organelle DNA is to encode some subunits of multi-subunit organelle proteins, the remaining subunits being encoded in nuclear DNA; (2) those protein subunits encoded in organelle DNA are synthesised by organelle ribosomes, whereas protein subunits encoded in nuclear DNA are synthesised on cytoplasmic ribosomes. For example, D. E. Griffiths (University of Warwick) reviewed data on yeast mitochondrial ATP-synthetase, which is composed of ten subunits; six of these are nuclear coded and made on cytoplasmic ribosomes, whereas the other four are made by mitochondrial ribosomes and are presumed to be encoded in mitochondrial DNA. The major chloroplast protein (fraction I protein or ribulose diphosphate carboxylase) is composed of large and small subunits. R. J. Ellis (University of Warwick) described the synthesis of the large, but not the small subunit, by isolated intact chloroplasts and by free chloroplast ribosomes. Tryptic peptide analysis of mutant tobacco plants has shown that the large subunit is encoded in chloroplast DNA whereas the small subunit is encoded in nuclear DNA. The emphasis of work in this area is now shifting away from the identification of products of the different genomes towards a study of the regulatory mechanisms existing between them.

Elegant studies of mitochondrial DNA isolated from cytoplasmic *petite* mutants of yeast were reported by P. Slonimski (CNRS, Gif-sur-Yvette); this DNA is derived from wild type DNA by large deletions which are replaced by reiterations of the non-deleted segments. The resulting gene redundancy can be detected by electron microscopic studies of half-denatured molecules. Stable *petite* mutants in which different parts of the mitochondrial DNA are repeated and amplified up to 100 times can be isolated, thus opening the way for the selective purification of different mitochondrial genes. Such studies on chloroplast DNA are much less advanced because of the difficulty of inducing mutations in the DNA of this type of organelle; R. Hagemann

(Martin Luther University, Halle) reported that some naturally-occurring chloroplast mutations in higher plants are correlated with the absence of specific thylakoid proteins associated with photosystems I and II, and other mutations result in a loss of chloroplast ribosomes.

All of the products of protein synthesis by mitochondrial ribosomes, and most of those by chloroplast ribosomes, are membrane-bound. This raises severe problems of purification and isolation, but these problems are being steadily overcome. In a *tour de force*, R. O. Poyton (University of Connecticut) reported the isolation in milligram quantities of each of the seven subunits of yeast cytochrome oxidase. Four of these are made by cytoplasmic ribosomes and are water soluble when isolated, but the other three are made by mitochondrial ribosomes and remain insoluble. The three insoluble subunits are synthesised by isolated mitochondria, provided that oxygen is present; in anaerobic conditions, only one subunit is made. The mechanism of this interesting effect is unknown, but could reflect a specific effect of oxygen on mitochondrial transcription or translation.

On the second day, further work in the area of organelle biogenesis was discussed. G. S. P. Groot (University of

Amsterdam) suggested from an analysis of base sequence homology in nuclear and mitochondrial DNAs of different yeasts that mitochondrial DNA evolves faster than nuclear DNA, and L. A. Grivell (University of Amsterdam) reported that yeast mitochondrial ribosomal RNA is extraordinary in its complete lack of ribose methyl groups and a very low level of base methylation compared with other ribosomal RNAs. A contribution on chloroplast RNA by M. R. Hartley (University of Warwick) was notable, in that it reported, for the first time, the detection of a specific messenger RNA from a plant source (other than a viral messenger). This messenger is for the large subunit of fraction I protein which can be correctly translated by a cell-free protein synthesising system from *Escherichia coli*. Studies of specific messenger RNAs from plants have lagged far behind those from animals, so this advance should open up a new area of plant molecular research.

Outstanding problems that were raised, but not resolved, were the function of the genes in chloroplast DNA, 90% of which are still unidentified, and the mechanism of the uptake of specific proteins into and across the outer membranes of mitochondria and chloroplasts.

Neural attenuation in echolocating bats

from our Animal Behaviour Correspondent

THE sonar system possessed by many bats enables them to perform such feats as flying unhindered through an array of fine wires and selecting one insect from a swarm of many. The echoes from surrounding objects must be perceived a few milliseconds after the bats have emitted an extremely intense outgoing sound pulse (often several thousand dynes per cm²). Somehow the bats' ears must be protected from their own cries and yet still be sensitive enough to pick up the much fainter returning echoes. There are at least two mechanisms for accomplishing this. The muscles of the middle ear contract synchronously with vocalisation (they can contract and relax again up to 60 times s⁻¹ on some species) and this has the effect of attenuating self-stimulation.

In addition to this peripheral mechanism, there are neural mechanisms of attenuation within the auditory pathways. Suga and Schlegel (*Science*, 177, 82; 1972) made tape recordings of the calls of vesperilionid bats and recorded the response of a bat's auditory nerve both to this tape recording

and to the same call when made by the bat itself. Although the response of the primary auditory nerves was virtually the same to both sorts of sound, the inferior colliculus (part of the mid-brain concerned with hearing) showed much less response to the live than to the tape-recorded sound. Attenuation, by some 25 dB, to the bat's own voice was occurring somewhere between the auditory nerve and this part of the brain.

Now Suga and Shimozawa (*Science*, 183, 1211; 1974) have pinpointed the exact site of this neural attenuation. By recording from various nuclei along the auditory pathway when bats of the genus *Myotis* were either vocalising themselves or listening to tape recordings, they found that neural attenuation occurs in the nucleus of the lateral lemniscus. They point out that although such mechanisms are of obvious importance for bats with their special problems in echolocation, similar muscular and neural attenuation mechanisms may also operate in human speech, since our vocalisations rarely appear disturbingly loud.

Nuclear physics at Glasgow meeting

from a Correspondent

At the combined Institute of Physics conferences on nuclear structure and high energy physics, which took place at the University of Glasgow on March 27-29, the nuclear structure talks reflected in the main the specific interests of Glasgow workers. Experimental work in Glasgow concentrates on electron scattering, photoreactions and the use of photoreaction neutrons. The principal local activity in nuclear theory is the application of the shell model to fairly light nuclei.

E. Spamer (Technische Hochschule, Darmstadt) discussed the techniques by which the momentum resolution of the Darmstadt linear accelerator is being improved by a factor of five. He emphasised that this makes electron inelastic scattering applicable to the study of a great many more states than hitherto, at higher energies and in heavier nuclei. One would, for example, hope to learn more about the newly detected quadrupole resonance found above the familiar giant dipole resonance in medium nuclei.

R. Owens (University of Glasgow) dealt with photonuclear reactions in the higher energy region in which one hopes to obtain information about high momentum components in nuclear wave functions. This hope has been much deferred, and calculations with short range correlations seem at present to call for a further extension of the energy range in these experiments.

Short range correlations were also a feature of the contribution by J. M. Irvine (University of Manchester) who dealt with low lying levels of the p-shell nuclei. He has used a two-body interaction fitted to scattering deuteron data and a variational method applied to wave functions with both central and tensor correlation corrections, to obtain an effective interaction for use in a large basis shell model calculation with the Glasgow code. With reasonable restrictions on the short range central correlation, and by adjusting the strength of the tensor correlation to fit the absolute binding energy of each nucleus in turn, many other levels can be fitted with no further adjustment of parameters.

The talk by R. Arvieu (University of Paris, Orsay) also touched on the subject of large scale shell model calculations. He discussed the statistical approach to the shell model, in which one calculates the mean energy and moments of the level density distribution for various configurations. This can be used, for example to learn, in advance of investing in a large shell model calculation, which configurations are most essential

and must be retained. H. A. Weidenmüller (University of Heidelberg) also discussed statistical aspects of nuclear physics, but in the more familiar context of the statistical model of neutron resonances. He emphasised that although statistical shell model calculations give the density of levels in a configuration in Gaussian form, as opposed to the semi-circle law of Wigner's random matrix model, the two models agree on the fine structure of the statistics of level spacings. The predictions of these models are well confirmed by data with up to 180 neutron resonances in a particular nucleus. Weidenmüller also discussed recent work at Heidelberg which extends the range of validity of the Hauser Feshbach prescription for average cross sections, and which allows effects of direct reactions to be allowed for in this method.

On the high energy side there was considerable emphasis on photon and lepton interactions although strong

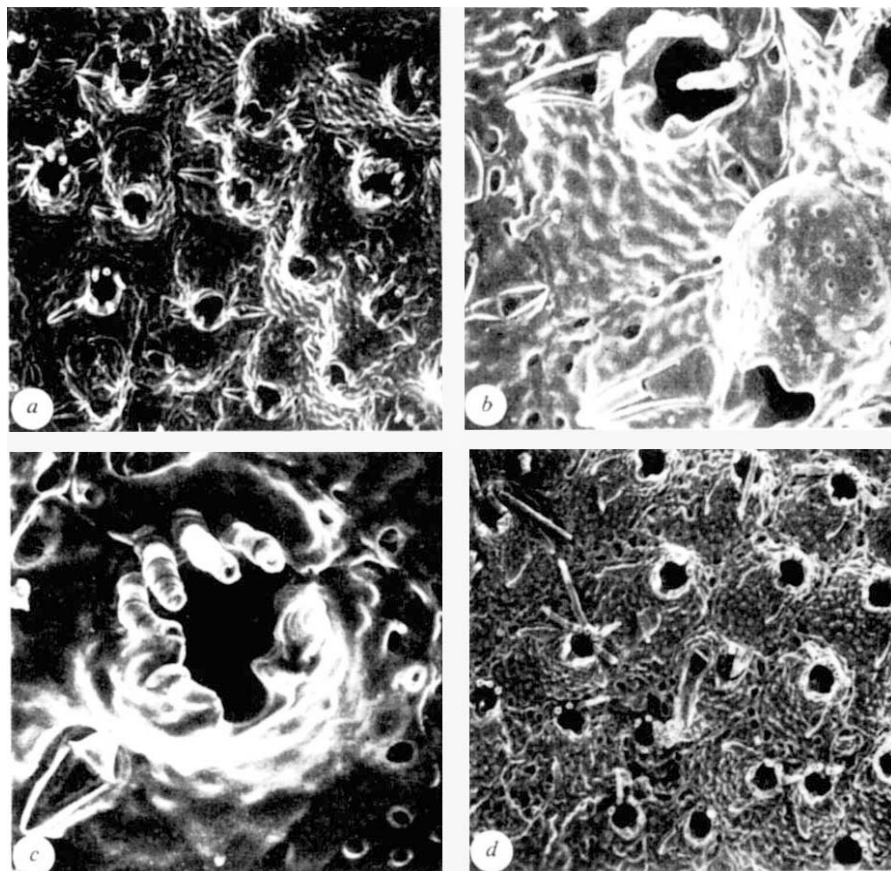
interaction physics, particularly at very high energies, also figured strongly. D. H. Perkins (University of Oxford) reviewed the experimental evidence for neutral currents in weak interactions and indicated that the experimental evidence is becoming steadily more convincing and is in good agreement with the Weinberg-Salam-Ward theory as opposed to the long standing Fermi theory which, as for example in beta decay, requires only charged currents.

Two examples of $\bar{\nu} e^-$ scattering have

been recorded in the Gargamelle bubble chamber at CERN and many more events of the type $\nu + \text{hadron} \rightarrow \nu + \text{hadron}$ at both CERN and the National Accelerator Laboratory, Batavia (NAL).

Storage rings—both electron and proton—are producing results of fundamental interest and several speakers concentrated on data recently produced by these devices which allow observa-

Smittinid ectoproc from Hawaii



The shallow waters round the Hawaiian Islands seem to be havens for the smittinid family of ectoproct bryozoans—the minute sedentary aquatic animals which usually live in colonies. D. F. and J. D. Soule have found that the Hawaiian species of Smittinidae seem to be isolated specifically from the Indo-Pacific forms and that there are also species differences between the five major Hawaiian Islands. In a comparative study (*Bull. Am. Mus. nat. Hist.*, **152**, 365; 1973), the Soules recognise twenty-eight taxa of which two are new genera and twenty are new species. This figure shows (a) colony with tubular peristomes, (b) immersed ovicell and part of developing ovicell and (c) aperture with spines of *Parasmittina alanbanneri* n.sp.; (d) colony with spines on zoecia of *P. serrula* n.sp.

tion of collision between particles at energies hitherto unattainable by conventional accelerators.

J. Haissinski (University of Paris, Orsay) spoke about electron-positron storage rings where e^+e^- collisions at centre of mass energies of several GeV are studied. From the study of such collisions two main conclusions may be drawn: the first concerns quantum electrodynamics—the fundamental theory which describes the interaction between electrons, muons and photons—which is found to be still valid at distances as short as a few hundredths of the proton radius. Second, electron-positron annihilation processes can lead to the production of hadrons (strongly interacting particles such as pions, protons, neutrons) and production rates have proved to be unexpectedly high. If this trend persists at higher energies it would have important consequences as far as 'elementary' constituents of the hadrons are concerned. These hypothetical fundamental constituents (quarks) are now the object of an intense programme. The storage ring experiments seem to indicate that either these quarks form a rather large family, or that they have a structure of their own. In the latter case, the situation would be quite similar to the one which resulted from the discovery of the internal structure of the nucleons in the 'elementary' constituents of the nuclei.

The experiments currently taking place at the intersections of the Intersecting Storage Rings at CERN were reviewed by M. G. Albrow (CERN). Searches are being made for new exotic particles which may be produced at the very high centre of mass energies available at the ISR (up to ~ 56 GeV). Searches for quarks, magnetic monopoles and intermediate vector bosons have all so far proved negative although antideuterons have been observed. The important discoveries at the ISR of a 10% rise in the total pp cross section and of structure in the angular distribution of elastic scattering are now being measured with higher precision and studies of interactions where particles are produced with high momentum transfer relative to the incident proton direction are also proceeding. The observed high rate of such events would indicate the existence of point like constituents (partons) within the nucleon.

The current interest in nucleon substructure was also reflected in talks by R. G. Moorhouse (University of Glasgow) who dealt with photo- and lepto-production processes in the context of the quark model and by F. Close (CERN) who described how the number of quarks in a particle can be determined by means of the energy dependence of the elastic scattering cross section at large angles.

Out of the ball park

from a Correspondent

At the Spring meeting of the British Biophysics Society, devoted to nucleation, folding and interaction of biopolymers, held at the University of Newcastle upon Tyne on March 26-28, and organised by Dr R. H. Pain of the Biochemistry Department, the policy was continued of devoting the major part of the programme to long (50 min) papers. In discussion the extent to which the various specialist techniques used to study biopolymers should be regarded as mutually complementary rather than competitive became very clear. A further impression was the way in which improvements in instrumentation and access to computers have facilitated more sophisticated interpretations of data from techniques such as ultracentrifugation and gel chromatography on complex systems of interacting biopolymers, and the rigorous testing of these interpretations by computer modelling of the experimental data.

R. L. Biltonen (University of Virginia) examined the thermodynamics of folding of biopolymers, with particular reference to the thermal cooperative transition to a partially unfolded state on an effective 2-state basis, and to the associated large change in heat capacity (ΔC_p). Large differences in heat capacity between conformational states of biopolymers is now well established, and can be attributed in part to large changes in hydrophobic bonding, and in part to changes in solvent (water) structure due to the rupture of internal hydrogen bonds by partial unfolding, and the formation of new hydrogen bonds between exposed side chains and solvent molecules.

S. Lifson (Weizmann Institute, Rehovot) appealed, with both eloquence and wit, for an increase in the precision of calculation of molecular properties from crystal structure data, arguing that results "in the ball park" were no longer of interest. Conversely, the more precisely that calculations agree with experiment the more interesting the remaining discrepancies become in pointing the way to modification of theoretical parameters. After demonstrating the success of the consistent force field (CFF) method for calculating the properties of the alkanes, Lifson reported a study of the hydrogen bond, starting from crystallographic data on several amides to find the best force field. In the final result charge transfer seems to be unimportant and a 12-6-1 potential, which combines the Lennard-Jones 6-12 potential with partial charges attributed to the participating atoms while keeping the bond electroneutral,

properly represents the hydrogen bond. There is a reduction in the effective van der Waals radius of the amide hydrogen, due to shifting of the electron cloud and formation of a large partial positive charge. This allows close approach of the $>NH$ and $O=C<$ groups to form a hydrogen bond of characteristic length, and the resulting associated electrostatic attraction is the main source of the bond energy. Lifson cheerfully conceded that this theoretical picture might have to be drastically modified to explain those features of hydrogen bonds revealed by infrared spectroscopy.

The extent to which the elucidation of hydrophobic bonding is dependent on a theory of water structure (still the subject of much argument) was emphasised by G. Némethy (University of Paris, Orsay) who pointed out that different agencies for changing hydrophobic bonding may alter the properties of proteins in aqueous solution in quite different ways.

B. Robson (University of Newcastle upon Tyne) discussed the application of information theory to estimate the extent to which sequence dictates secondary structure in the folding of a polypeptide chain. It seems that specific single residues can act as nucleation centres for secondary structure, and that the conformation of segments of the polypeptide chain are thus determined locally by the constituent residues. In the refolding process the locally ordered segments may represent metastable intermediates formed early in refolding and surviving in the stable completely folded conformation. Robson noted that Ramachandran conformational energy maps based on Lifson CFF functions were better than most, and also drew attention to map regions for the 4-residue reverse turn, which is now being recognised as an important element of secondary structure.

G. C. K. Roberts (National Institute for Medical Research, Mill Hill) described how NMR spectroscopy can be used to study protein unfolding, and referred to work on pancreatic ribonuclease (RNase). Subject to the difficulties of assigning NMR resonances to protons in individual residues, which is currently possible only for a small number of particular residues in the proteins studied in detail, it is possible in principle to follow the participation of individual residues in unfolding processes in reversible conditions. For RNase, the NMR studies indicate that the thermal transition at pH 5 proceeds cooperatively to a still partly ordered state, and that two of the four histidine C_2 proton resonances are affected. With denaturants such as guanidine hydrochloride, the NMR spectra at low denaturant concentration

indicate changes not revealed by optical methods. From resonance peak areas as a function of denaturant concentration, it seems that the order in which the histidine residues participate in the unfolding process is 12, 119, 48, 105. This order can be rationalised in terms of the known tertiary structure of RNase, and is therefore also relevant to the folding process.

The possible methods for revealing the existence of intermediate partially unfolded states were noted by R. H. Pain who pointed out that although some earlier equilibrium and kinetic studies on, for example, lysozyme unfolding in guanidine hydrochloride, indicate a 2-state process with first-order kinetics up to about 90% reaction, this situation is not general. The analysis of bimodal kinetics can reveal the presence of intermediate states, or of alternative states off the main folding-unfolding path. Pain reported his investigation of the penicillinase of *S. aureus*, a protein with no sulphhydryl or disulphide groups, which was specifically selected for unfolding studies, and summarised the evidence for the existence of a highly expanded state, with the same α -helix content as the native protein, formed by a cooperative transition at low denaturant concentrations.

L. W. Nichol (Australian National University, Canberra) discussed ligand binding in associating protein systems, and showed how the form of the binding curve could be discussed in terms of regions of sigmoidality defined by the interaction parameters. He described how ultracentrifugal sedimentation equilibrium measurements can be used to evaluate these parameters. G. A. Gilbert (University of Birmingham) emphasised the central role of protein-protein interactions in the conformational control of protein function mediated by allostery, and pointed out that in the so-called non-equilibrium methods (for example, chromatography, electrophoresis, velocity centrifugation) the progress of the moving boundary reflects the equilibrium situation in the following plateau region, as well as the non-equilibrium situation within it.

The topical subject of membrane-bound proteins and protein-lipid interactions was discussed by C. Tanford (Duke University). Experimental data on protein-detergent associations (much used as a contemporary model) were described. The behaviour of cytochrome *b5* and a phage protein in detergent systems as consistent with the idea that membrane proteins possess distinct regions around which true micellar structures can organise. The discussion included a brisk exchange on the problems of measuring protein molecular weights by ultracentrifugation in the presence of detergents.

Proton NMR studies on histones were

discussed by E. M. Bradbury (Portsmouth Polytechnic). With increasing ionic strength structure is induced through interchain interactions between regions of apolar residues, and the basic segments interact with DNA, leading to the formation of chromosome superstructure. The lysine-rich F1 histone, however, seems to be involved specifically in salt-induced contraction of chromatin gel, and in chromosome condensation through a phosphorylation process.

The programme included two parallel sessions of shorter papers on various aspects of protein unfolding and folding processes, ribosome reconstitution, RNA-protein complexes, and mucoprotein. Noteworthy was the study by Creighton, Dyckes and Sheppard (MRC Laboratory of Molecular Biology, Cambridge) on the refolding of reduced pancreatic trypsin inhibitor, in which the different species existing at the 1-disulphide bond stage were detected and characterised. The refolding of this protein proceeds with eventual complete recovery of native structure, and the pathway for refolding indicated by this work is consistent with the view that folding is directed by segments which have acquired the correct secondary structure.

Chlorophyll and primary productivity

from our Plant Ecology Correspondent

SINCE chlorophyll is essential for photosynthetic energy conversion, it is not unreasonable to suppose that it might provide a rough estimate of the potential gross primary productivity of an ecosystem. Early in this century the work of German plant physiologists suggested a close relationship between chlorophyll and photosynthetic rate. Later it became apparent that the chlorophyll density of vegetation expressed as g m^{-2} of ground area was remarkably similar in a variety of ecosystems and this led Odum (*Fundamentals of Ecology*, Saunders, 1959) to propose that there is a community homeostasis in which chlorophyll levels are maximised within the limiting physical constraints of the environment, leading to similar chlorophyll densities in most ecosystems.

Such a tenuous hope was shattered by Bray (*Can. J. Bot.*, **38**, 313; 1960) who concluded that chlorophyll densities in a wide variety of vegetation types might vary by as much as twenty times in natural ecosystems. He found that chlorophyll densities were higher in climax ecosystems than in seral ones and were greatest in highly stratified systems such as forests. These conclu-

sions were largely confirmed by Ovington and Lawrence (*Ecology*, **48**, 515; 1967) who made detailed studies of four contrasting ecosystems—a prairie grassland, a savanna, an oakwood and a maize field.

Although Odum's simple homeostasis proposal was no longer tenable, there remained the possibility that chlorophyll might provide an index of gross primary productivity. Ovington and Lawrence's data in no way contradicted such a proposal. The underlying presupposition, however, is that all of the chlorophyll present must be active in photosynthesis whenever light levels permit; physiologists have always regarded this as extremely unlikely.

One rather startling observation which Ovington and Lawrence made was that a large proportion of the total chlorophyll content of their oak woodland was contained in the branches of the trees. In the month of August the trees themselves contained $29 \times 10^3 \text{ g ha}^{-1}$ chlorophyll, of which $12 \times 10^3 \text{ g ha}^{-1}$ (41%) was contained in the older branches. The bulk of this branch chlorophyll remained after leaf fall in September/October. Immediately the question must be raised as to whether this large reserve of chlorophyll is active and, perhaps even more important, whether it could result in winter photosynthesis in these deciduous trees.

Although only relatively simple experiments were required to settle this question, they have been conducted only very recently. Much is known about the winter photosynthesis of evergreens (see, for example, Kozlowski and Keller, *Bot. Rev.*, **32**, 293; 1966) but very little work has been done on deciduous trees. Keller (*Photosynthetica*, **7**, 320; 1973) has now examined rates of photosynthesis and respiration in the bark of ash (*Fraxinus americana*), aspen (*Populus tremula*) and larch (*Larix decidua*) during the winter. In his experiments plants were taken from their growing sites where they had been exposed to normal winter temperatures, they were kept at 16°C for 16–20 h and their carbon dioxide exchange rates were then measured by infrared gas analysis at 40, 10 and 0 klx respectively. In all species at all light intensities the carbon dioxide emission exceeded absorption; in other words, respiration exceeded photosynthesis. Thus, despite the presence of abundant chlorophyll, these species are likely to be experiencing a negative carbon balance through the winter months.

These experiments should serve as a salutary warning to production ecologists of the dangers inherent in the use of chlorophyll level as a productivity index, despite the general correlation between chlorophyll density and the gross primary productivity of ecosystems.

DNA-mediated genetic correction of thiamineless *Arabidopsis thaliana*

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Thiamineless mutants of Arabidopsis were corrected by treating the seeds with DNA preparations from various bacteria. Possible mechanisms for this are discussed.

TRANSFORMATION of prokaryotes by addition of exogenous genetic information has been possible since the classical experiments of Avery *et al.*¹ and since the demonstration that bacteria can take up and integrate DNA into their genome by genetic recombination.

Until recently, such 'cell manipulation' with higher organisms seemed almost impossible, presumably because of the inadequate means available for critical studies. The necessary biochemical background had to be obtained: good methods to prepare nucleic acids and to recognise them in a population of similar molecules had to be developed. The scarcity of mutants with defined biochemical lesions precluded (and still limits) genetic experimentation^{2,3}.

Nevertheless, DNA-mediated corrections in higher organisms were reported: with mammalian cells in tissue culture⁴⁻¹², with *Ephestia*¹³ and with *Drosophila*^{14,15}, with *Petunia*¹⁶⁻¹⁹ and with *Arabidopsis thaliana*²⁰⁻²⁵. Finally, phage-mediated corrections were reported with animal cells²¹, with haploid tomato and *Arabidopsis calluses*²⁶⁻²⁸ and with sycamore cells²⁹.

Extensive use of the CsCl gradient technique has led to the unexpected results that foreign DNA can be translocated to large distances across cell membranes^{30,31} and seems to survive several mitotic cycles in different animal or plant systems^{2,30,32}. In *A. thaliana*, it seems to survive at least one round of meiotic division^{23,25,33}. More surprisingly, it seemed that the foreign DNA became covalently linked to the recipient DNA^{21,23,25,30} as double-stranded material (a phenomenon similar to the incorporation of the λ DNA in the *E. coli* genome).

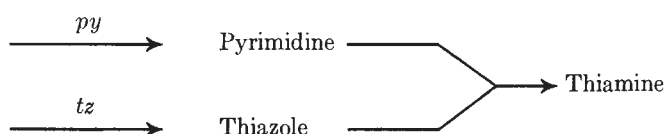
As already indicated in preliminary reports^{20,25}, correction of nutritional mutants of *A. thaliana* by exogenous DNA was attempted. We present here an overall picture of the results so obtained. More extensive genetic data will be presented elsewhere.

Nutritional mutants of *Arabidopsis thaliana*

Described auxotrophs are rare in *A. thaliana*, with the exception of those requiring thiamine³⁴. They can be obtained by X rays or chemical mutagens. They are also conditional obligate auxotrophs with a strict phenotype, whilst other auxotrophs exhibit bradytrophs.

Mutants at one locus (*py*) control the synthesis of the pyrimidine moiety of thiamine^{34,35}, locus *tz* controls the

synthesis of the thiazole half of the vitamin and two other genes are concerned with some intermediate steps between these two precursors and thiamine³⁴. These mutants can be used for strictly genetic studies such as allelic complementation or recombination or reverse mutation because of the built-in selective mechanisms³⁴. The mutability of the *py* locus is apparently the highest^{34,35} and is close to 2×10^{-4} . The frequency of spontaneous reversion has been estimated to be lower than 5×10^{-6} .



Treatment with ethyl methanesulphonate produced apparent reversions at the *py* locus, at a frequency of about 4×10^{-5} . These revertants were nonhomozygous and segregated in the following generations. In test crosses, they also yielded heterozygotes and lethal mutants in approximately equal frequencies³⁶. No homozygous wild types were thus obtained by back mutation.

The monogenic conditional lethal mutants used here are normally propagated in the presence of thiamine. When sown on mineral medium, the seeds germinate normally and develop their cotyledons which appear normal green. The first leaves progressively bleach out completely and the plant soon dies. Thiamine (10^{-6} M– 10^{-5} M) promotes a normal growth of all these mutants.

Mutants used in this study were the following: *thi51* from Rédei, *thiV447* and *pyV131* from Feenstra, and *py431* and *tz432* from Jacobs. They bear the side markers indicated in Table 1; the only wild-type *Arabidopsis* used in the laboratory was the Wilna *gl*.

Treatment of seeds

Seeds were disinfected with a 95% ethanol and 3% H_2O_2 mixture (1:1) or with a 5% calcium hypochlorite solution³⁴. The seeds were treated for 5–8 min and rinsed with a few changes of sterile distilled water. About 50 seeds were then immediately placed in a well of a spot plate and immersed in 25 μ l of a DNA solution (see below) or of 0.01 M NaCl. Spot plates were placed in Petri dishes over wet sterile filter paper to limit evaporation of the solvent. During the three following days, 25 μ l of the DNA solution were added to the seeds to keep them moistened. Petri dishes were kept at 24° C under continuous illumination for 4 d. Both DNA and NaCl treated seeds germinated during the incubation period. After incubation seedlings were laid out (a) in tall Petri dishes containing perlite moistened with the mineral nutrient solution of Jacobs³⁷ or (b) in numbered test tubes containing 5 ml 0.78% agar dissolved in the mineral nutrient solution

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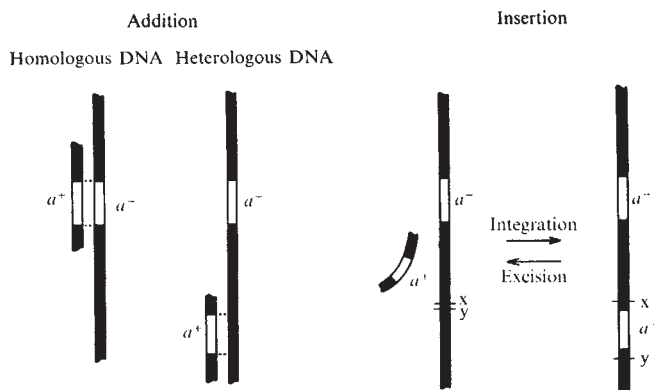


FIG. 1 Comparison of addition and insertion models (one of two homologous chromosomes is shown). In *Drosophila* transformed by homologous DNA Fox's exosome model implies a close association of the foreign gene with its homologous chromosome locus. This exosome is not integrated but replicates in step with the chromosome and can be lost or transmitted with it at the time of cell division. Either the exosomal gene or the chromosomal one is transcribed, leading to phenotypic mosaicism. When heterologous DNA is used for correction, as in our case, the necessity of a close association with the mutated site is less mandatory and the heterologous exosome could become associated to another region, where the actual base sequence is in favour of such an association. Here too, the exosome could replicate in step with the chromosome and be lost or transmitted with it at the time of cell division. The probability of a loss would however be high due to the imperfect homology. In the insertion model, the foreign gene is integrated in the recipient DNA, like episomal DNA, through the homology of a few base pairs. Once integrated, it would be hard to lose it, except on pairing with a chromosome homologous for the rest of the structure but lacking the inserted foreign piece. Although gene conversion could interfere at that step, chromosome aberration is an obvious alternative, possibly leading to the loss of the foreign gene.

of Miksche and Brown³⁸. The tubes were placed in wooden racks, kept under continuous illumination (6,000 lux) at 24° C and examined each second day.

All mutant seedlings on mineral medium died within 15–20 d; they showed chlorophyll deficiency in the cotyledons and in the rosette leaves. Corrected plants grew slowly, with a more or less normal green colour. About one-third produced fertile siliques (see below). These siliques were collected and kept separately in cellophane bags. The thiamine concentration necessary to provide complete growth when seeds were soaked in it for 3 d, then washed and allowed to develop in the absence of thiamine, was found to be about 10^{-3} M.

DNA preparations

DNA preparations were obtained from frozen or fresh bacteria (or from isolated and CsCl-purified bacteriophages) by the technique of Marmur³⁹ or by CsCl gradient preparative ultracentrifugation, after treatment with pronase and RNase⁴⁰. The DNA was precipitated with 2 volumes of ethanol, kept in 70% ethanol, and dissolved in sterile 0.01 M NaCl just before use. The concentration used was about 0.5–1.0 mg ml⁻¹. We successively tried and rejected the use of chloroform or phenol for deproteinisation: both are toxic to the plant. They may also selectively remove the protein or membrane bound DNA molecules from the bulk of the DNA preparation.

Our study of the biochemical fate of labelled DNA in various systems led us to recognise that the DNA has to be freshly prepared, of high molecular weight ($> 10^7$ daltons), very clean and intact. When single-strand breaks in a DNA preparation could be detected from the depolymerising effects of alkali⁴⁰, the preparation was discarded. Finally, the DNA had to be given during the first 24 h of imbibition of the seeds; otherwise it was not integrated into the recipient material (unpublished data).

In general, we used small numbers of seeds per batch of DNA and preferred to repeat the treatment with freshly prepared batches. Perhaps because of fragmentation of the DNA molecules during their isolation and purification, some batches of a given DNA species were ineffective with the mutant plants tested, whereas others were quite effective. Yet these DNAs were undistinguishable by the physicochemical methods used. Even if it is tempting to blame the DNA itself, it is of course difficult to eliminate uncontrolled parameters related to plant growth as the source of these discrepancies.

DNA was prepared from the following strains: *Escherichia coli* K12, wild type; *Agrobacterium tumefaciens* B6, obtained from Dr A. Kurkdjian; *Bacillus subtilis*, 168 T⁻; *Micrococcus lysodeikticus*, ATCC 4698; *Streptomyces coelicolor* AS/2 obtained from Dr H. Hopwood; *E. coli* P678 (*thiaA*). Phage T7 (with normal bases) and phage 2C (with hydroxymethyl uracil replacing thymine) were also used. They were isolated and purified following the technique of May *et al.*⁴¹

Correction in DNA-treated populations

When seeds of the different mutant types were treated with different bacterial DNAs, corrections could be obtained with a relatively high frequency (10^{-2} to 10^{-4}) comparable with that observed with bacterial transformation. Table 1 shows an overall picture of the data gathered during the last 3 yr with a total of 27 different batches of DNA tested with five different mutants. These data include DNA extracted from wild-type bacteria, from bacteria with a thiamine deficiency (corresponding to the thiazole pathway) and from phages containing normal (T7) or abnormal (2c) bases.

Further controls were obtained by treating mutant seeds with 0.01 M NaCl, the solvent used for the DNA assays. After DNA treatment, a few mutant seedlings, which normally died within 15–20 d of sowing, displayed better growth, formed small rosettes and eventually flowered. Only a few of them were fertile, however. Table 1 takes into account only the restored plants which produced seeds, thus allowing a study of their progeny. Table 1 also indicates, for each case, the number of germinating seeds and the total number of seeds used. Contamination by wild-type seeds is highly improbable as strict precautions were taken in treating and growing the plants. Furthermore, most of the mutants used bear additional markers which make them easily recognisable from the only wild-type strain used in the laboratory. These results indicate that the percentage of correction obtained with the group of DNAs from wild-type bacteria is similar for all mutants: 0.7%, 0.85% and 0.49% respectively for thiamine, thiazole, and pyrimidine-requiring mutants. On the other hand, the percentages of correction obtained for five mutants seem to vary with the source of DNA, *E. coli* being at the moment the most effective (0.89%) and *S. coelicolor* the less effective source of DNA (0.19%). Negative results were obtained with DNA from phage T7 or phage 2C. DNA from *E. coli thia* did not correct thiazole mutants but, as expected did correct pyrimidine ones. It should also be noted that in all cases, the DNA-treated plants, growing in the absence of thiamine showed a delay in growth and development, as compared with the wild type or to mutants supplemented with thiamine (5×10^{-5} M).

Progeny obtained by selfing corrected types

Progenies of various corrected types obtained after DNA treatment were sown on perlite soaked with mineral medium or on soil and observed during the entire growth of the plant. The number of F1 plants tested varied as a function of the number of seeds present in the fruits of the corrected plant growing on a thiamineless medium. In a very few cases, thiamine was provided at the flowering stage to increase vigour and thus seed production. F2 and F3 progenies in-

TABLE 1 Corrections observed in DNA-treated populations

DNA	<i>thi</i> 51 (g1)	<i>thi</i> V447 (er-g1)	Lethal mutants (additional markers)		<i>py</i> 431 (-)	Total
			<i>tz</i> 432 g1	<i>py</i> V131 (er, g1)		
a.						
<i>E. coli</i>	6/355/685	2/393/525	2/284/550	1/384/400	4/270/477	15/1686/2637
<i>Agrobacter tumefaciens</i>	2/182/420	0/100/100	3/230/480	1/100/200	0/130/250	6/742/1450
<i>Bacillus subtilis</i>	0/128/288		3/157/328	0/28/100	0/162/420	3/497/1136
<i>Micrococcus lysodeikticus</i>	0/176/450		0/122/120		2/428/705	2/428/1375
<i>Streptomyces coelicolor</i>	0/145/274	1/75/75	0/152/272	0/22/100	0/120/410	1/524/1131
b.						
<i>E. coli</i> P678* (<i>thi</i> A ⁻)			0/263/350	1/254/350		1/517/700
Phage T7 (<i>E. coli</i>)	0/425/542		0/440/500		0/356/520	0/1221/1562
Phage 2C (<i>B. subtilis</i>)	0/189/252		0/182/220			0/371/472
c.						
NaCl 0.01 M	0/395/642	0/233/291	0/467/669	0/214/262	0/1580/2960	0/2889/5024

Figures show no. of corrected plants with a progeny/no. of germinated seeds/no. of treated seeds.

* Requiring thiazole for growth.

cluded at least fifty plants for each experiment. The majority of the corrected plants were studied up to three successive generations and fresh and dry weights for 5 corrected mutants were determined.

The most striking result of this study concerns the lack of segregation on selfing in the progenies of the various corrected types. All F1 to F3 plants originating from each corrected plant look phenotypically alike and grow reasonably well on mineral medium. This was observed also in one case where the selfing was pursued up to the seventh generation. They show, however, in most but not all cases, a variegated pattern of chlorophyll pigmentation with light discolouration at the base of the leaf and along the main vein. In practically all cases, the average fresh weight (and particularly the average dry weight) of the corrected types grown on mineral medium, were lower than those of plants from the same progeny, supplemented with 10^{-4} M thiamine (37 to 92%). Each of these types thus display sensitivity to the addition of thiamine. Other experiments showed that the corrected 'pyrimidine' or 'thiazole' mutants specifically responded to the addition of pyrimidine or thiazole and showed an improved growth. On the other hand, and contrary to the situation for wild-type control plants, the progeny of some of the corrected plants exhibit thermosensitivity; at 16° C they showed signs of thiamine deficiency, which disappeared at a higher temperature.

In selfing experiments, the corrected plants behaved as homozygotes breeding true, in contrast to what is found with mutagen-induced revertants. This is also in contrast to what is expected in the case of a suppressor mutation introduced into a homozygous diploid. Indeed, in the seed, the germline is represented by two diploid cells^{42,43}. These cells, at least, should contain the thiamine information to explain the lack of segregation after selfing. In F1 the ratio of mutants: wild types should vary from 1:3 to 1:7. The fact that correction results in a homozygous corrected plant suggests that there has been continuous selection for the corrected germ cells and, presumably a meiotic drive at the time of meiosis favouring those gametes which harbour the thiamine information. Indeed, when selection pressure is avoided by giving thiamine supplementation to plants treated with DNA which show signs of abortive flowering, a high percentage of lethal mutants is observed in the F1 progeny.

Offspring of out-crosses

We have analysed, in parallel, the offspring of crosses made between corrected plants and either the wild or the original mutant type (back or test crosses). Table 2 shows the results obtained in the case of *tz*^a a homozygous 'thiazole' mutant corrected by a treatment with *B. subtilis* DNA. (Similar data were and are being gathered for other corrected types such

as *py*^a, *th*^a or *tz*^a corrected with other DNA. They will be described elsewhere together with the results of crosses between mutants corrected with different DNA). The phenotypes presented by progenies of successive self fertilisation were analysed.

The first generation of the outcrosses (x) does not segregate. The results of the test cross (*tz*^a × *tz*⁻) implies that the correction is dominant and that the corrected plants behave as true homozygotes. Reciprocal crosses between corrected mutants and lethal mutants indicated that the correction can be transmitted through the male as well as through the female and therefore does not seem to correspond to a maternal cytoplasmic factor.

Leaky mutants

Segregation is observed in the offspring of the corrected plants outcrossed to the wild type (xF1 → xF2). Lethal mutant types are recovered (although in low frequencies) together with plants exhibiting a new phenotype called here 'leaky mutant'. This is characterised by a weaker pigmentation, white patches of discolouration on the leaves, sometimes white sectors on the stem and relatively poor growth and fertility, in contrast with the normal looking plants of the same progeny. A whole range of these phenotypes, intermediate between the normal green plants, and the lethal chlorophyll deficient plants were classified under the name 'leaky mutant'. These mutants are obtained in crosses between the corrected type and the wild type, as well as in crosses between the corrected type and the lethal mutant (in the latter case, a high frequency of lethal mutant being obtained). The leaky types keep their sensitivity to supplementation with thiamine, thiazole or pyrimidine respectively. This indicates that the correction has been added to the mutation and has not been substituted by it. Correction being dominant, the recessive mutation is not expressed unless the correction is masked or lost, for instance by crossing with an uncorrected plant.

The leaky mutants are found at equal frequencies in the XF1 offsprings from *tz*^a × *tz*⁺ (9.2%) and *tz*^a × *tz*⁻ (6.1%). Their offspring includes high frequencies of leaky and lethal types and also normal looking plants. This normal phenotype can be recovered at frequencies (21.7% and 24.7%) suggesting that the 'leaky mutants' behave as heterozygotes. They should however, also segregate into mutant types with similar frequencies. The correspondingly lower values obtained for the lethal mutants and the reproducibility of the phenotype distributions could be accounted for by various mechanisms.

We expected that this study would help to correlate genetic and biochemical data. This was in fact the main reason why bacterial DNA was used in the biological studies. We have not used homologous *Arabidopsis* DNA because it

TABLE 2 Results of outcrosses and control crosses

Cross	Progeny tested	No. growing plants	No. ungerminated plants	Normal (N)	% Phenotypes Leaky (Lk)	Lethal
$tz^c \times tz^+$	$\times \rightarrow \times F1$	11	20	100		
	$\times F1 \rightarrow \times F2$	163	15	90.1	9.2	0.7
	$\times F2N \rightarrow \times F3$	198	5	95.5	2.0	2.5
	$\times F2Lk \rightarrow \times F3$	198	13	21.7	70.2	8.1
$tz^c \times tz^-$	$\times \rightarrow \times F1$	20	5	100		
	$\times F1 \rightarrow \times F2$	116	15	75.8	6.1	17.3
	$\times F2N \rightarrow \times F3$	131	4	76.3	0	23.7
	$\times F2Lk \rightarrow \times F3$	202	21	24.7	65.0	10.3
$tz^- \times tz^+$	$\times \rightarrow \times F1$	15	0	100		
	$\times F1 \rightarrow \times F2$	106	7	72.6	0	27.4
	$\times F1 \rightarrow \times F2$	63	4	73.0	0	27.0

is difficult to prepare from this material the clean, intact DNA of high molecular weight which we consider a prerequisite for efficient uptake and integration. On the other hand, although we wanted to use specialised transducing phages to introduce selected genes only, we were not in a position to do so, because of the lack of phages carrying thiamine information. (The amount of DNA which can be taken up by a cell is limited as are the size of the populations to handle. It therefore seems reasonable to try to increase the dosage of the interesting gene in the DNA preparation used. This can be achieved by using DNA from bacteria, transducing phages or episomes.) This kind of difficulty has recently been overcome by using DNA from thiamineless bacteria (ineffective as such for correction) harbouring a F' factor bearing the thiamine information (KLF10). These results will be published elsewhere. To what type of genetic model do these results lead?

Transformation models

Different models have been discussed by Fox *et al.*¹⁵ to explain their results showing genetic transformation in *Drosophila* with homologous DNA. They argue in favour of the exosome model, also considered by Hess to interpret *Petunia* transformation. The exosome model (Fig. 1 of ref. 22) is the only one which does not require an integration of the exogenous information into the linear structure of the chromosome. Such an integration is rejected by Fox because of the absence of whole-body transformants among the transformed *Drosophila* and to the instability of the correction. In the *Arabidopsis* system the nonautonomous type of biochemical lesions arising from the possible diffusion of thiamine makes it difficult to ascertain the presumably mosaic nature of the corrected type. Its progeny is, however, easier to analyse. It therefore seems that the corrected plants do behave as homozygotes as they do not segregate in their offspring (they do not segregate 1:1 in test crosses). As the correction is stable in subsequent offspring, it must correspond to a surprisingly stable form of exosome.

The effect of the exogenous DNA seems to be associated with the gene concerned (thiamine, thiazole or pyrimidine loci). This leads to the concept of an association of the bacterial information with the *Arabidopsis* genome. Such a close association is also indicated by the absence of significant differences between progenies of reciprocal crosses implying corrected types; 100% normal plants are obtained upon crossing corrected either male or female to the mutant type. The thiamine mutation remains in the genome of the corrected type; by crossing the corrected plants with the wild type, a resurgence of the original mutant type does occur in F_2 although at very low frequencies. In fact, the passage through meiosis for the plants arising from a cross with one of the corrected types seems to increase the instability of the exogenous information. This seems to be the consequence of the appearance of the 'leaky mutant' phenotype (which could eventually be considered as functional

mosaics).

The regularity in the transmission of the genetic information as well as the survival of the mutated site in the corrected plant could be explained if we assume that the necessary amount of information has been added to the genome (and not substituted for the mutant gene) and can be removed by crossing the plant with the wild type or with the mutant, but not on selfing. Perhaps difficulties in chromosome pairing, because the correction is present in only one of the two homologous chromosomes forming the bivalent, could be responsible for removing this correction. Such a picture fits well with the 'episomal DNA' type of interpretation (Fig. 1 of ref. 22) emerging from the biochemical analysis. This model also allows an explanation for the variability in the functioning of the bacterial thiamine gene in the recipient plant, possibly related to its relative position. These differences in the phenotypic expression of the thiamine locus do not seem to result from a lack of integration but from a change in the gene environment that is able to influence its genetic transcription and therefore its expressivity.

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LETTERS TO NATURE

PHYSICAL SCIENCES

Perturbations of Pioneer 6 telemetry signal during solar occultation

ATTENTION has been drawn recently to unexplained perturbations in the telemetry signal of Pioneer 6 (2,300 MHz) during solar occultation. The results¹ shown in Fig. 1 present the following odd features:

(1) An anomalous redshift is added to a normal linear redshift due to the spacecraft oscillator. This residual redshift which is symmetrical with respect to the centre of the Sun is of the order of $z = 5.18 \times 10^{-8}$ at four solar radii.

(2) The bandwidth increases sharply when the telemetry signal grazes the Sun.

(3) There are some extremely sharp pulses in the bandwidth. In Fig. 2 we show that these pulses are clearly associated with a sharp increase of the redshift and correspond to solar flares.

The existence of the redshift is particularly puzzling because it cannot be attributed to gravitational effects nor to the usual Doppler effect.

Various explanations of those phenomena have been attempted:

Photon-photon interaction: P. Merat and colleagues⁸ (unpublished work) have presented an explanation based upon previous work by Pecker, Roberts and Vigier^{2,2a}. Assuming an hypothetical photon-photon interaction, they supposed that each photon passing through a photon bath loses energy according to the semi-empirical law:

$$z = -\delta\nu/\nu = C \int U(s) ds$$

where $U(s)$ represents the background photon density, s the distance travelled and C a constant whose value lies around $10^{-30} \text{ cm}^2 \text{ erg}^{-1}$. Their hypothesis gives a good account of the redshift. But the width of the signal remains unexplained, as does the correlation with solar flares; indeed the photon density $U(s)$ remains constant during the flare. And the

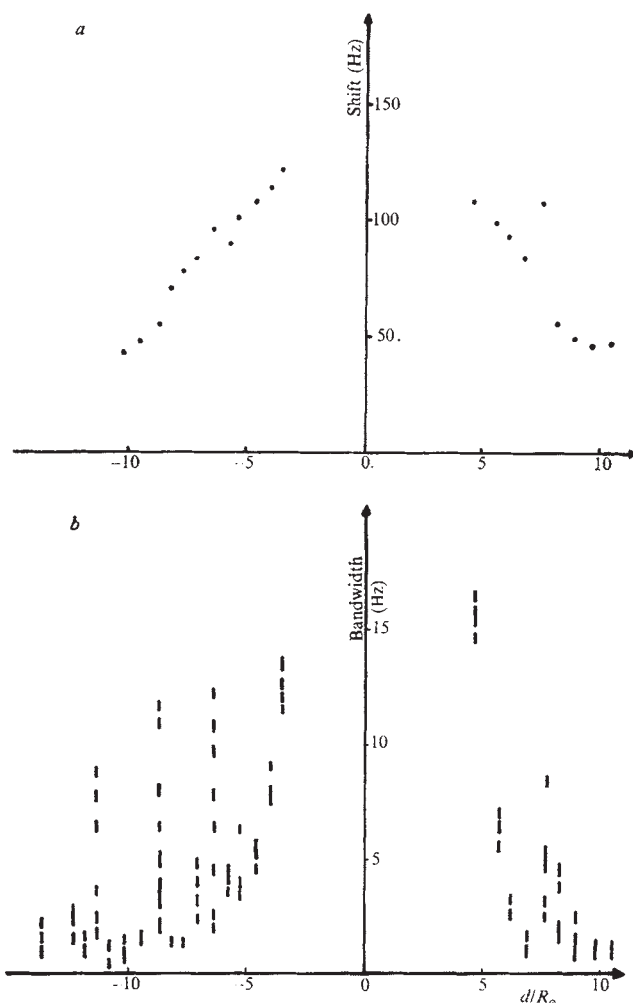


Fig. 1 Residual redshift (a) and bandwidth (b) in Hz as functions of the minimum distance of Pioneer 6 telemetry signal to the centre of the Sun.

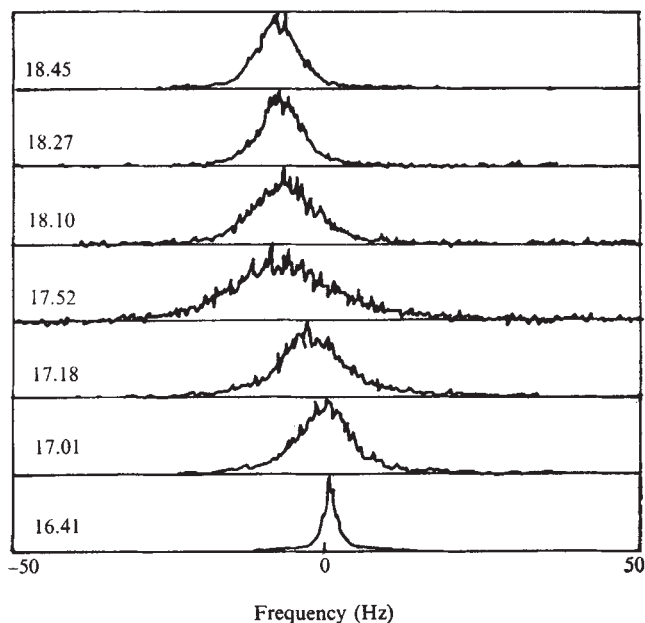


FIG. 2 This sample spectrogram taken on November 8, 1968, shows the effects of a solar event and demonstrates the association between the broadening of the line (12 Hz h^{-1}), the anomalous redshift (4 Hz h^{-1}) and the solar flares.

theoretical foundation of their formula is, in our opinion, unclear. We have therefore considered more 'classical' explanations.

Doppler-type effect: The optical index n varies when the signal passes through the solar corona. But it is easy to see that this effect is too small (of the order of 2.5×10^{-9}) and asymmetrical with respect to the Sun.

Induced scattering: It is well known that induced scattering gives rise to a redshift of intense electromagnetic radiation if the scatterers have a decreasing energy spectrum. This is true for ions and electrons. But calculations including an energetic tail in the distribution function of the electrons indicate that the effect is much too small. Scattering which might change the nature of the waves, in particular Langmuir into electromagnetic waves, is inefficient at this frequency.

Non-linear wave interactions: The combination and decay of waves or scattering of waves by waves must be considered. A plasma can support electromagnetic waves, space-charge waves and low frequency waves of the acoustic type. But interaction of electromagnetic waves with high frequency (Langmuir) waves leads to the build-up of a system of satellite lines, at a distance from the central lines equal to the plasma frequency ω_e , that is much larger than the frequency window in Goldstein's experiment. Similarly, scattering of these waves would introduce both a large angular scattering and a frequency broadening of the order of the plasma frequency.

Therefore, low frequency waves are the only ones that may give rise to the perturbations.

We emphasize that if such processes are responsible for the perturbations of the signal, it is simple to explain why these perturbations are correlated with solar flares, which are known to generate strong electronic perturbations in the solar corona. Low frequency waves in the solar corona can be either ion-sound waves or magneto-sound waves depending on the magnetic field.

Evidence for the existence of ion-sound waves in the solar corona are of three types: (1), Gordon³ explained strong radar backscatter from the Sun using them; (2), scintillations of radio sources allow us to study the density fluctuation and therefore the level of turbulence $W_{\text{turb}}/W_{\text{th.e}}$ in the corona, at least at high altitudes; (3), broadening of sources of solar origin (such as Type I and Type III bursts⁴) indi-

cates that density fluctuations are certainly present.

From all these data we estimate that at four solar radii the level of turbulence is of the order of a few per cent while the frequency ω_e of the ion-sound waves could be of the order of 0.1 Hz.

Our calculations have shown that induced processes are negligible and that the decay process (e.m. wave $\rightarrow$ e.m. wave + ion-sound wave⁶) gives essentially a broadening of the line:

$$\Delta\sigma^2 = \frac{1}{512\pi} \frac{\omega_e^4 \omega_e}{\omega^2} \frac{L}{c} \frac{c_s}{c} \frac{W_{\text{turb}}}{W_{\text{th.e}}}$$

where $W_{\text{turb}}/W_{\text{th.e}}$ is the level of turbulence and L the interaction length between the solar plasma and the telemetry signal. At four solar radii, ω_e and c_s/c have to be taken respectively as 10^7 Hz and 3×10^{-4} , leading to a broadening of the line of the order of 10 Hz as observed. Further, the scattering process (e.m. wave + ion-sound wave $\rightarrow$ e.m. wave + ion-sound wave⁷) could very well produce a redshift. Detailed calculations of this effect will be presented elsewhere.

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Speeding up astronomical photography

VARIOUS methods have been used for increasing the sensitivity of photographic emulsions prior to exposure in the telescope. For example, washing red sensitive (panchromatic) emulsions in ammonia or water, and latterly baking blue sensitive (orthochromatic) emulsions at 50° C have given reductions in the exposure times required to reach a given density, although at the cost of greater intrinsic fog and non-uniformities, and poorer photometric properties. More recently, placing the photographic plates in vacuum for several hours, with or without simultaneous baking, has come into common use for some emulsions, and this has been followed by baking in a dry nitrogen atmosphere (see ref. 1 and references therein).

The object of baking or vacuum treatment is to remove from the emulsion water vapour and oxygen, which act as desensitising agents. Both techniques depend on the principle of excess partial pressures to drive out the unwanted gas or vapour, in the first by increasing the partial pressures in the emulsion and in the second by reducing them in the ambient atmosphere. Both produce undesirable side effects, the first an increase of intrinsic fog, often non-uniform, the second the removal of all gases and a shrinkage and hardening of the emulsion.

It seemed to us that the objective could be achieved on the basis of excess partial pressures but without introducing undesirable side effects, by prolonged soaking of photographic plates in oxygen-free dry nitrogen of high purity, at room temperature and pressure. So we carried out experiments in the laboratory at Edinburgh in mid-1972, photographic plates being stored in a gas tight tank which

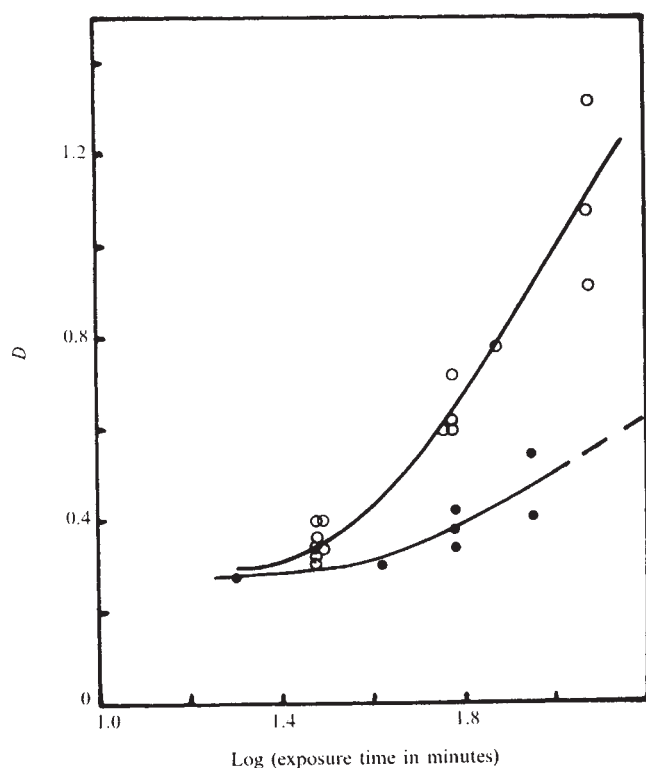


FIG. 1 Photographic diffuse density against logarithm of exposure time in minutes. Open circles, nitrogen sensitised IIIaJ emulsion; filled circles, unsensitised.

was flushed with high purity nitrogen for several minutes each day. These experiments showed that, in the case of the Kodak IIIaJ emulsion, nitrogen soaking for a month was necessary and gave reductions by factors of between two and five in the exposure times needed to reach moderate density—a similar result to that obtained by vacuum baking but without the undesired increase in intrinsic fog.

Prolongation of the soaking gave no further gain but no deleterious effects resulted. Delaying exposure of the plate for 24 h after removal from the nitrogen showed no significant fall in the sensitivity that had been gained—a not unexpected improvement over the alternative baking and vacuum techniques.

These preliminary results were communicated privately to astronomers in Britain, at the European Southern Observatory and at Palomar, and to Eastman Kodak.

The nitrogen soaking technique has been in routine use with the $f/2.5$ UK 48-inch Schmidt Telescope at Sliding Spring, Australia since the telescope was commissioned in July/August 1973. Here we report on the efficacy of the technique in operating conditions; this is important because it is well known that the photometric properties of photographic emulsions generally depend on the temperature and humidity prevailing during exposure in the telescope. The results refer to Eastman Kodak IIIaJ plates (batch 2C3) since this emulsion type is to be used for the Science Research Council's southern sky survey.

Figure 1 shows the densities reached with given exposure times in the telescope for 15 sensitised and 7 unsensitised plates. The sky brightness during the exposures was $B = 22.25 \pm 0.1$ mag (arc s) $^{-2}$, measured by a photoelectric photometer. The densities measured by a Macbeth densitometer are close to ASA diffuse values.

There were considerable reductions in the exposure times required to reach given densities, by a factor 2.5 at $D = 0.6$ and increasing with density (Fig. 1). Such a gain would, however, be spurious if the photometric properties of the emulsion had shown a corresponding deterioration. It is

therefore important to compare the density noise before and after sensitising, and this is done through the factor

$$(D/\sigma_D)^2 \quad (1)$$

where D is the density and σ_D is the r.m.s. density noise for the sampling aperture used, in this case $25\mu\text{m} \times 35\mu\text{m}$ at the plate. The quantities were measured for nine sensitised and six unsensitised plates and show no significant difference between the two sets.

It is notable that any increase of intrinsic fog density due to the sensitising was ≤ 0.05 .

A measure of efficiency sometimes used in the USA is

$$\text{overall efficiency} = \left(\frac{dD/dE}{\sigma_D} \right)^2 S \quad (2)$$

where E denotes exposure and S the reciprocal exposure required to reach a specular density of 1.0 (probably close to $D = 0.6$ in this paper). The results produced here for the nitrogen soaking technique show a gain by a factor of about 20 in this measure at $D = 0.6$, about equal to the gain reported for baking in nitrogen.

Using sensitised IIIaJ plates, limiting magnitudes have been determined by photographing the star cluster Kron 3 which contains a photoelectronographic sequence to $B = 22.7$ by Walker². These show that $B = 22.3$ is detected in 30 min, $B = 22.7$ is visible in 1 h, and easily visible in 2 h. A reproduction of a 2 h exposure is shown in Fig. 2. For comparison, an optimum 40 min exposure on IIaO emulsion reaches $B = 21.5$.

The nitrogen soaking technique has been tested on Kodak IIaO emulsion by B. A. Peterson and J. McKinlay at the

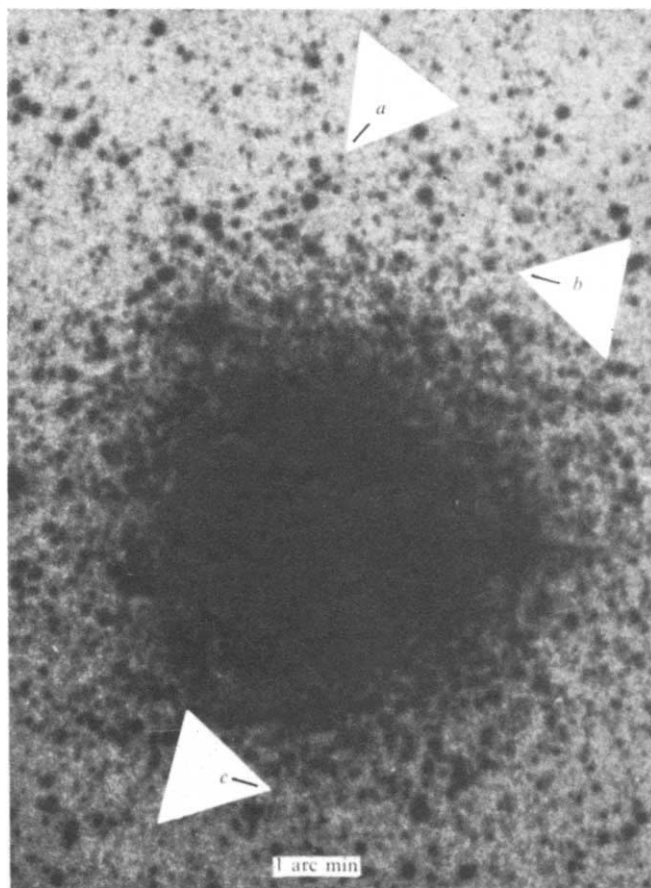


FIG. 2 Enlargement of the star cluster Kron 3 in the field of the Small Magellanic Cloud. Exposure 2 hours on nitrogen sensitised IIIaJ plate with the UK 48-inch Schmidt telescope. According to Walker's photoelectronographic measurements, the stars marked *a*, *b* and *c* are respectively of magnitudes $B = 22.7$, 22.3 and 21.7 .

Mt Stromlo Observatory, and they report the same gain as for baking but without the undesirable increase in intrinsic fog.

The large gain in efficiency obtained by nitrogen soaking of the Kodak IIIaJ emulsion has led to it being used for the Science Research Council's southern sky survey which will consequently have a limiting magnitude $B = 23$. This is two magnitudes fainter than the Palomar survey of the northern sky which was carried out before this emulsion was developed.

Another emulsion with the high signal to noise characteristic of the Kodak IIIaJ is the Ilford R40. This panchromatic process emulsion was brought into use by one of us (V. C. R.) at the Royal Observatory Edinburgh in 1956 and has since been used regularly there and by the UK 48-inch Schmidt Telescope in Australia. It has good photometric characteristics, low noise and high uniformity. Nitrogen soaking does not affect its sensitivity. It is difficult to compare its performance with that of the IIIaJ because of the three times wider bandwidth of the R40 which has a peak sensitivity in the red, and because the telescope corrector plate is optimised for the blue waveband giving better images on the IIIaJ, but one hour exposures of the same field taken with sensitised IIIaJ and unsensitised R40 plates show that most objects occurring on either plate occur on the other.

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Molecular hydrogen in X-ray astronomy

THERE is a serious discrepancy between determinations of the column density of interstellar matter in the direction of the Crab Nebula. Virtually every soft X-ray observation¹⁻⁵ with sufficient energy resolution to derive this column density yields a result substantially in excess of determinations based on 21-cm absorption⁶⁻⁸. The quality of the X-ray and radio data is now such that this disagreement must represent a genuine physical effect rather than an artefact of the data analysis.

It seems likely that this situation also applies to many or all galactic X-ray sources. The discrepancy is easily recognised in the Crab as it is a strong source with a well defined, nonvariable, power-law spectrum extending over many decades, but many other low latitude X-ray sources show a similar effect⁹, as does a recent detailed study of the Tycho supernova remnant¹⁰. If the cause of this anomalous absorption is interstellar, it will affect all distance estimates of X-ray sources based on correlated 21-cm and soft X-ray observations.

So far there is no satisfactory explanation of this phenomenon. An increased abundance of heavy elements over that assumed in the X-ray photoelectric absorption cross section^{11,12} has been considered^{1,2,4}, but the range of elements responsible for absorption in the 0.5-1.5 keV band is limited. Oxygen is eliminated¹ as the required absorption edge does not appear prominently in X-ray spectra. Helium cannot be solely responsible⁴ since the required abundance increase would be hopelessly inconsistent with all other data. A substantial increase in the Brown and Gould¹² neon abundance coupled with a modest increase in helium could reconcile the data⁴ but the latest estimates of the cosmic neon abundance¹³⁻¹⁶ are at variance with this explanation.

Absorption due to interstellar grains with an extreme superabundance of carbon has also been examined¹. An increase of the cosmic carbon abundance by an order of magnitude above currently accepted values is required. Carbon is one of the few reasonably well-determined heavy element abundances, so this seems unlikely.

I suggest that interstellar molecular hydrogen is responsible for a significant component of the necessary opacity. The predominance of molecular hydrogen in dark interstellar clouds is well understood theoretically and established observationally, and the very low abundance of H_2 in regions of negligible optical extinction also presents a consistent theoretical and observational picture. But the intermediate case of the fractional H_2 abundance in interstellar clouds of low to moderate optical extinction is not nearly so well studied or understood, and this is in fact the case appropriate to the Crab Nebula.

The optical extinction in the line of sight to the nebula has been determined by several methods¹⁷⁻¹⁹ whose results agree quite well and centre on $A_V = 1.8$ mag. In this region of moderate extinction there have been detections of both hydroxyl²⁰ and formaldehyde (C. Heiles and T. Troland, personal communication; R. Davies, personal communication) in absorption against the Crab continuum source. These observations indicate two distinct molecular clouds which correspond well with neutral hydrogen features in both velocity and profile^{6,7}.

The chief problem is whether clouds of such moderate optical depth provide sufficient shielding from the interstellar ultraviolet radiation field so that any significant H_2 abundance may be expected in an equilibrium situation. I examine the feature⁶ at 11 km s⁻¹ and, since two molecular clouds are observed, assume that it provides about half the observed visible extinction, implying an optical depth of the cloud centre in visible light²¹ of 0.4. It could be argued instead that the extinction increases in a completely linear fashion over the distance of 2 kpc, although the available data are against this possibility¹⁸. This, then, is an assumption of the model, although hardly an extreme one, and the X-ray data itself is evidence in its favour. Using the model parameters of ref. 6 and the shielding criterion of Hollenbach *et al.*²², I find that H_2 can be formed for fractional molecular abundance of $f \gtrsim 0.8$. This is in fact close to the abundance that is observed by OAO for some stars with comparable visual extinction²³. Under these assumptions, this cloud could contain $N(H_2) \geq 1.4 \times 10^{21}$ molecules cm⁻². Although interferometric studies²⁴ have shown small gradients in optical depth across the face of the radio source, the X-ray source is of much smaller spatial extent²⁵ and so all this material should be available to attenuate the X-ray spectrum.

This argument is conservative in that the direct observation of hydroxyl and formaldehyde is alone compelling evidence for the presence of a substantial amount of H_2 . The nominal lifetime of these molecules in clouds of moderate optical depth is extremely short, which strongly implies a deficiency in the theoretical calculations of molecular formation or destruction rates, or both, in the sense that larger fractional abundances than predicted are invariably observed^{7,26}. In particular, Herbst and Klemperer²⁷ have noted that all reactions which effectively produce formaldehyde require molecular hydrogen.

Can the inferred amount of H_2 provide the missing opacity? Unfortunately the photoelectric absorption cross-section of H_2 at soft X-ray energies is not well established, with theory¹² and experiment²⁸ in some disagreement between 0.1 and 10 keV, but calculations and experiments agree on the somewhat surprising result that the H_2 photoelectric cross section substantially exceeds twice the atomic cross section, apparently because of molecular bonding effects. There are no measurements at precisely the wavelengths of interest but Brown and Gould¹² estimate a cross section

of 180 b per molecule at 1 keV, giving the cloud under consideration an X-ray optical depth at this energy of $\tau \geq 0.25$. The amount of 'missing matter' generally derived by most experimenters^{4,5} is of order 1×10^{21} atoms cm^{-2} or an optical depth $\tau = 0.3$. Thus the H_2 in this cloud alone may make a substantial contribution to the necessary opacity. Since both the theoretical and experimental values of the cross section increase more rapidly than that of the overall interstellar medium as one considers energies < 1 keV, even the smallest molecular hydrogen cross section recently suggested²⁸ will supply sufficient opacity at 0.5 keV to explain the entire discrepancy.

This sharp increase of the cross section at longer wavelengths also enables us to predict that the apparent opacity discrepancy should become worse for observations at softer energies. Thus a positive detection of the Crab source at 0.25 keV, which may be within present capabilities, would provide a test of this proposal. It may also be possible to observe the H_2 directly in absorption against bright stars with small angular displacements from the Crab using satellite-borne equipment. The colour excesses of the closest such stars, Zeta Tau and 114 Tau, indicate however that they are probably not behind the same cloud as the nebula itself.

There are several problems using correlated 21-cm and soft X-ray data to set distances to galactic X-ray sources²⁹. Intrinsically variable and self-absorbed X-ray spectra, uncertain cross sections, abundances, spatial abundance gradients and interstellar medium excitation temperatures all contribute errors. If we now include the possible presence of non-negligible amounts of H_2 in the direction of many galactic X-ray sources it seems that, contrary to the conclusions of several previous investigations, such correlations may not be useful as accurate distance indicators.

The hypothesis presented here may have possible implications for observations of the soft component of the diffuse X-ray background. These molecular clouds of low-to-moderate optical depth are most easily recognised in absorption against a few strong radio sources but if they are present elsewhere in any significant number they could cause substantial small-scale granularity in the observed X-ray flux. This granularity will not necessarily be well correlated with 21-cm data over small regions of sky and could lead to the erroneous conclusion³⁰ that the observed flux is not of extragalactic origin.

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Kilometre and centimetre Earth strainmeters

It has been customary to construct strainmeters within a length range of 5–500 m (ref. 1). Prior to the instruments reported here the longest instrument was a laser strainmeter 1,020 m long² and the shortest also a laser instrument 1 m long³.

It has been recognised for many years that long strainmeters are desirable for the measurement of strains near the Earth's surface. There are two principal advantages: first, a long strainmeter averages out short wavelength variations in the strain field; second, the signal to be measured increases proportionately with instrument length whereas the 'attachment noise', caused by instabilities in coupling the instrument to the ground, remains constant. The long instrument approach using a laser system has been successfully operated above the Earth's surface⁴.

The advantages of short instruments lie in their portability and ease of installation if suitable low noise methods of mounting them to the rock can be used.

The instruments described here are mechanical, remarkably simple and inexpensive. The long instrument is 1,025 m long (thus the longest strainmeter ever built) and the short instrument is 45 cm long and is the shortest ever built. The

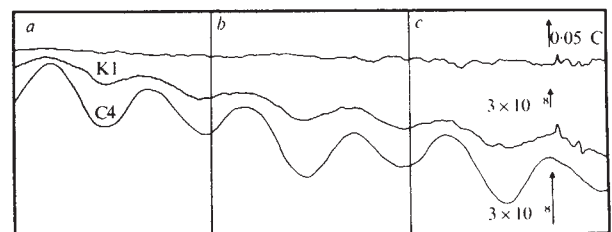


Fig. 1 Three days of data from Queensbury Tunnel, Yorkshire. a, September 13; b, September 14; c, September 15, 1973. K1 is a 1.025 km wire strainmeter and C4 a buried 10 m wire strainmeter. The upper trace is from a thermistor thermometer. Earth strain extension upwards.

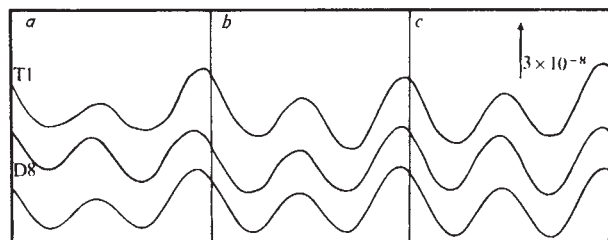


Fig. 2 Three days of data from Schiltach Observatory, Schwarzwald. *a*, January 15; *b*, January 16; *c*, January 17, 1973. The upper trace is from a 45 cm strainmeter (N20E) and trace D8 is from a nearby 10 m wire strainmeter (N2E). The lower trace is the predicted tidal strain (N2E). A linear trend of 3×10^{-8} strain contraction per day has been removed from the observed data.

two strainmeters have operated well for 6 and 12 months respectively.

As a length standard the 1,025 m strainmeter uses a 0.38 mm diameter Invar 36 wire held under constant tension. The wire is supported at approximately 9 m intervals along its entire length by thin wires which constrain vertical and transverse motions but allow longitudinal movements. At each end the strainmeter is attached to the gravel floor of the tunnel by a 20 kg steel plate resting on a thin bed of moist sand. At one end the wire attaches directly to the plate and at the other end a simple weight and lever system provides a constant tension of approximately 15 N. The principle of the tensioning system is the same as for other wire strainmeters developed at Cambridge^{5,6}. The resonant modes of the instrument are complex; the amplitude of the fundamental resonant frequency (approximately 0.05 Hz) is reduced by mechanical damping and electronic filtering. A micrometer at one end provides a means of calibrating the instrument but this is only to within 20% due to uncertainties in the restoring force produced at each support point.

We made no attempt to protect the length standard from its environment. Consequently, some parts of the wire are subject to water dripping from the roof and others are exposed to air currents near imperfectly sealed ventilation shafts. Figure 1 shows three days of data. The upper trace records air temperature near the end of the long strainmeter. The lower trace is from a standard 10 m wire strainmeter enclosed in a pipe 50 cm below the tunnel floor. The remaining record is from the long wire strainmeter. Solid Earth tides are clearly seen on this instrument and it is obvious that some of the noise correlates with temperature changes. The higher noise level of the long instrument is not unexpected in view of its exposure to the air, an aspect of the design which clearly can be improved.

An interesting feature of multiple-support wire strainmeters is that within reasonable limits the instrument need not be straight. This ability to tolerate slight lateral or vertical offsets avoids the rigorous site constraints associated with other forms of long strainmeter.

The 45 cm strainmeter uses a 1.5 mm diameter Invar 36 wire as a length standard which is sufficiently stiff to support itself horizontally. The instrument spans a block joint in granite within the Geowissenschaftliches Gemeinschaftsobservatorium at Schiltach in the Schwarzwald, Germany. The purpose of the installation was to observe any anomalous tidal movements between the granite blocks but it has been found that tidal amplitudes are normal. The instrument is attached at each end by an expanding rock bolt. At one end the wire is bolted directly to the granite and at the other end the wire is fastened to an electromagnetic displacement transducer. The calibration of the system is estimated to be not better than $\pm 10\%$. The output is recorded on a multi-channel chart recorder together with four standard 10 m strainmeters. Three days of record are shown in Fig. 2, together with the predicted tidal strain for Schiltach.

The 45 cm instrument gives a clear tidal record. Its azimuth is 18° different from the 10 m instruments and data from all of these instruments have been spectrally analysed⁷. The amplitude agreement between instruments is within 10% at both diurnal and semi-diurnal periods. The close agreement of the 45 cm strainmeter with the 10 m instruments is surprising since alignment and attachment errors are correspondingly larger in the small device. In some circumstances it is conceivable that an array of colinear small instruments may provide a more representative value than a single larger instrument.

It is possible to use inexpensive mechanical devices as strainmeters with both very short and very long spans. There is no reason to suppose that it is impractical to build strainmeters with dimensions beyond the limits of those described. Whether this ability will prove useful or not is a separate and complicated problem which will only be resolved by experiments with large numbers of instruments.

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Production of secondary ice particles during the riming process

Ice crystals play a vital part in the formation of precipitation, particularly outside the tropics, so it is important to know the concentrations of ice particles in natural clouds and to attempt to predict them from measurements made, for example in laboratory cloud chambers, at the same temperature.

There are sometimes large discrepancies, however¹; for example² in maritime cumuli the concentration of crystals can exceed the concentration of ice nuclei as measured in a cloud chamber by a factor of 10^4 at -10° C. One of the oldest explanations³ for this, revived at intervals^{2,4}, is that ice particles growing by sweeping up supercooled drops might throw off secondary 'splinters' of ice. Recent laboratory experiments have, however, failed to show any appreciable splinter production in the riming process (ref. 5 and S.C.M., J. L. Brownscombe and G. J. Collins, to be published). We now present new experimental evidence that copious secondary ice particles are thrown off when ice particles grow by riming in clouds at about -5° C. This phenomenon, which occurs under a narrow range of conditions not covered by previous investigators, will probably explain the anomaly.

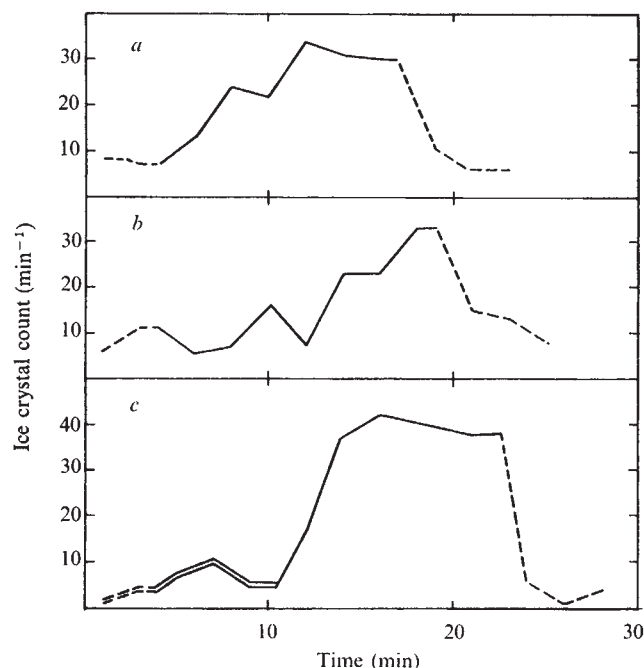


FIG. 1 Production of ice crystals by riming at $-5 \pm 1^\circ \text{C}$ ---, Target rod stationary; —, target rod moving at 2.7 m s^{-1} . *a*, Typical experiment with target rod in normal condition; *b*, Target rod coated with glycerin; *c*, Target rod heated above 0°C during period when curve is shown double.

Our experiments were carried out in a plastic walled chamber $1.5 \text{ m} \times 1.5 \text{ m} \times 1.7 \text{ m}$ high, enclosed in a cold room. A supercooled cloud of reproducible characteristics was made by injecting steam into the chamber from a boiler run at constant power. Rime was grown on a vertical metal rod 30 cm long and 0.24 cm in diameter which could be moved in a circular path of diameter 30 cm about a vertical axis, at velocities up to about 3 m s^{-1} , in the cloud. The bottom of the riming rod was 64 cm away from the steam inlet at closest approach. Ice crystals could readily be detected by eye in a light beam which traversed the chamber below the riming rod, provided one looked towards the light source at about 10° above or below the beam. With the rod stationary a few crystals per minute appeared in the beam. When the rod was rotated so as to sweep up water droplets which froze and built up as rime on the leading edge, the rate at which crystals appeared increased, to reach an approximately constant value after 4 min . When rotation was stopped, the count of crystals per minute returned to the 'background' value within 4 min . A typical experiment at a cloud temperature of -4.5°C is illustrated in Fig. 1*a*.

Two types of experiment demonstrated that the increase in ice crystal count was indeed caused by the riming and not by some other process, such as the detachment of ice fragments from the walls of the cloud chamber. In the first type of experiment, illustrated in Fig. 1*b*, the target rod was coated with glycerin. The ice crystal counts stayed small for the first 8 min of rotation, then increased when the glycerin had been sufficiently diluted for the supercooled drops to freeze and build up a layer of rime on the rod. In the second type of experiment, illustrated in Fig. 1*c*, the target rod could be heated by an internal electric heater to a temperature above 0°C . No rime grew on the moving rod and the crystal counts increased only slightly above the background value. When the heater was switched off and rime grew on the rod, the counts increased in a way similar to those shown in Fig. 1*a*.

The relationship between the rate of appearance of ice crystals in the light beam and the total rate of production of

crystals was established by placing trays of supercooled sugar solution at various positions on the floor of the cloud chamber and counting the number of crystals appearing in the sugar in a given time. A rate of appearance of one crystal in the beam per minute corresponded to a total production rate of $12 \text{ crystals per second}$. The rate of production of secondary crystals (after subtracting the background) was then divided by the rate of accretion of rime to give the number of crystals per milligram of rime.

Figure 2 indicates how the rate of production of ice particles varies with cloud temperature and shows that splinter production is virtually confined to the temperature range -3°C to -8°C . These experiments were carried out at a target velocity of about 2.7 m s^{-1} . As the velocity was decreased, the rate of production of splinters per unit weight of rime increased to a maximum and then fell away to zero at about 0.7 m s^{-1} (Fig. 3).

At a cloud temperature of about -5°C the density of the rime increased with velocity from 0.1 g ml^{-1} at 0.7 m s^{-1} to 0.5 g ml^{-1} at 2.7 m s^{-1} .

By attaching a glass slide, 3 mm wide and coated with MgO , to the riming rod and rotating it at 2.7 m s^{-1} in our experimental cloud at -5°C , we have established that the maximum drop diameter is $35 \mu\text{m}$ and that such drops are found in a concentration of 0.2 cm^{-3} .

The liquid water content of our experimental cloud was about 1 g m^{-3} , as indicated by an instrument in which the liquid water was evaporated and the resultant dew point measured.

Present experiments shed little light on the physical mechanism by which splinters are produced. The greatest rate of production occurs when the rime surface, being slightly warmer than the cloud, is at about -4.5°C . This is close to the temperature at which ice crystals growing by diffusion show a maximum in the growth rate along the c axis and grow as needles or hollow columns⁶. Drop interaction with these extreme crystalline forms may be responsible for splinter formation.

These experiments simulate the growth of graupel particles falling at constant orientation in a supercooled cloud. During the accretion of 1 mg of rime (corresponding to a graupel particle of diameter about 2 mm) several hundred daughter particles may be shed. If each secondary ice particle grows and rimes in its turn, there should be no difficulty in provid-

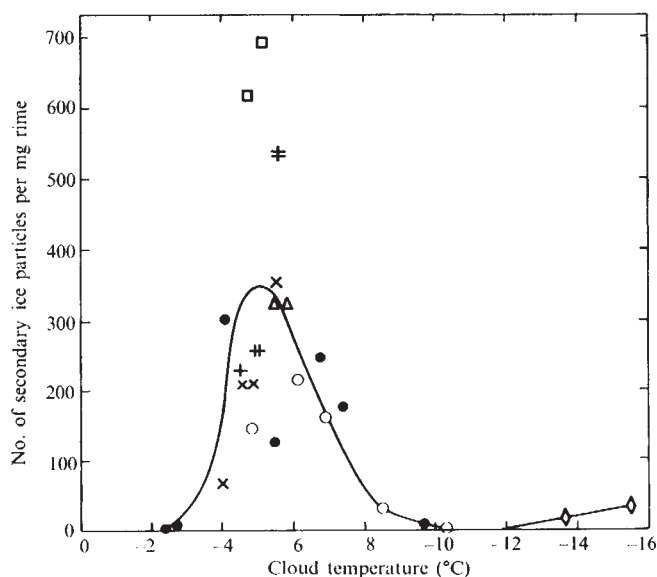


FIG. 2 Production of secondary ice particles by riming as a function of temperature at a target velocity of 2.7 m s^{-1} . Different symbols indicate different days. The curve was drawn by averaging the points over narrow temperature intervals.

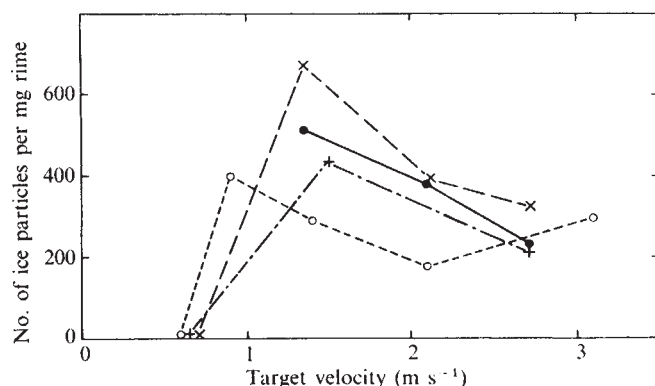


FIG. 3 Production of secondary ice particles by riming as a function of target velocity at a temperature of about -5°C . ●, November 5, 1973; -4.5°C , November 9, 1973; -5.5°C , December 3, 1973; -5.7°C , December 5, 1973; -5.2°C .

ing, within a reasonable time, the observed 'multiplication' factor of about 10^4 by which ice crystals sometimes exceed ice nuclei.

We have still to explain why the multiplication process is absent in some natural clouds which span the critical temperature range -3°C to -8°C . In the shallow stratiform clouds sampled by Mossop *et al.*⁷, this can be readily explained by the absence of rimed ice particles. The same argument cannot, however, be applied to the winter cumuli over Israel studied by Gagin⁸. These clouds contained plenty of rimed ice particles and also drops up to $36\text{ }\mu\text{m}$ in diameter. Yet there is little evidence of splintering, since the concentrations of ice crystals and ice nuclei agree to within a factor of 10. It may be possible to explain this on dynamical grounds. Maritime cumuli which show comparatively large crystal concentrations are usually wholly below the -10°C level², so that much of the graupel growth occurs at temperatures favourable for splintering. In the more vigorous clouds over Israel where the tops reach much colder temperatures, the initial riming growth of graupel particles takes place mainly at temperatures below -10°C . These particles may then fall rapidly, with surface temperature raised by latent heat release. Splinter production apparently requires a surface temperature of about -5°C and therefore depends not only on the ambient temperature of the cloud but also on the fall velocity of the particle and the liquid water content of the cloud⁹. In the Israel clouds the time for which the surface temperature of the graupel particles is close to -5°C may be so short that few secondary ice particles are produced.

We see that the high ice crystal concentrations observed in some cumuli can be explained without postulating earlier growth of the clouds to higher levels¹⁰: such growth may actually produce smaller concentrations.

The electrical effects accompanying splintering have still to be investigated; they may be important in charge generation in thunderstorms.

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Ultra-thin conducting films of gold on platinum nucleating layers

We report a method for obtaining highly conducting films of noble metals between 11 and 50 Å thick on mica substrates. At the lower limit, the ultra-thin film (11 Å) may consist only of two or three monolayers each of gold and platinum. The minimum thickness at which pure gold films start to conduct is known to be 30 Å (ref. 1). Before this we had found that a pre-nucleating layer of silver, 5 to 7 Å thick, on mica yielded a highly conducting gold overlayer above a total (Au + Ag) thickness of about 32 Å (ref. 2). It was concluded that nucleating layers of suitable materials, of a thickness below which island formation commences, might lead to a higher conductivity in films of total thickness less than 50 Å. In this range, irregularities in the surface of the substrate, for example cleavage steps, become comparable to, or even greater than the film thickness. Cleaved mica surfaces are known to have hill-and-valley type microstructure. The cleavage step heights are about 20 Å or integer multiples thereof³. As film thickness decreases to these values, d.c. measurements of conductivity become progressively less accurate. To alleviate this difficulty, we have measured microwave sheet resistance (R_s in Ω per square) at 9.7 GHz by Slater's method⁴. The corresponding wavelength is more than six orders of magnitude greater than any irregularity in the surface of the mica. We have used this method previously to estimate the conductivity of gold films thicker than 50 Å (ref. 5).

For the work reported here, platinum was selected as the nucleating material; adhesion of platinum to substrates is known to be better than that of silver^{6,7}. The films were deposited on to air-cleaved and clear mica sheets less than 0.001 inch thick, from a multi-crucible electron-beam evaporator. The substrates were kept at room temperature ($\sim 22^{\circ}\text{C}$). Eight targets were placed around the axis of the vapour beam at 50 cm from the source in a manner calculated to deposit films of equal thickness⁸. The mass-thickness of the films was monitored by a quartz crystal microbalance with the AT-cut, 5 MHz crystal placed in the centre of the targets. The crystal plate (0.75 inch-square, 1 mm thick) has a mass-frequency proportionality constant, C_f , of $57.6\text{ Hz cm}^2\text{ }\mu\text{g}^{-1}$ (ref. 9) giving a sensitivity of $11.13\text{ Hz }\text{\AA}^{-1}$ for gold and $12.36\text{ Hz }\text{\AA}^{-1}$ for platinum; the bulk and film densities were assumed to be the same. The oscillator frequency was measured by a digital frequency counter and recorded on a chart through a digital-to-analogue converter to ascertain frequency stability. With the crystal seat temperature stabilised by flowing water, the maximum frequency drift recorded over a period of 12 h was $\pm 5\text{ Hz}$. At an electron-beam power between 500 to 600 W, the deposition rate was typically $0.5\text{ }\text{\AA s}^{-1}$ for both metals. Films were thus deposited in a few seconds but, because the effects of thermal shock¹⁰ lasted more than 5 min, continuous monitoring of rate and

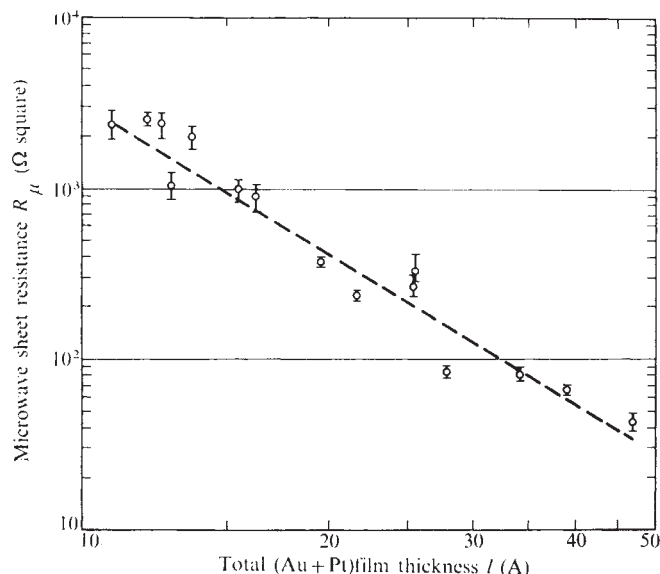


Fig. 1 Microwave sheet resistance, R_μ , against total (Au + Pt) thickness, l . — — —, $R_\mu = 2.77 \times 10^6 l^{-2.944}$ with a correlation coefficient of 0.96.

thickness was not possible. Deposition times were estimated and the difference between the stable pre- and post-deposition frequencies was used to compute the actual film thickness. The starting vacuum was $< 10^{-7}$ torr; the pressure rose to $\sim 5 \times 10^{-6}$ torr during deposition.

Using this method, gold films of varying thicknesses were deposited on to platinum nucleating layers ranging in thickness from 3 to 10 Å. A mean value of R_μ for sets of eight films deposited simultaneously is plotted against the total (Au + Pt) thickness, l , in Fig. 1. The adjusted standard error in R_μ is shown for each set of eight films; l is taken to be accurate within the limits of oscillator stability, that is ± 0.5 Å.

The conduction in these films was found to end abruptly for values of l less than 10.8 Å. Combinations of gold and platinum thicknesses to lower this limit by even 1 Å were not successful. It may, however, be noted that the sticking coefficient, α , of the evaporated films decreases rapidly with increasing lattice mismatch between the substrate and evaporant materials¹¹. α also depends on thickness and approaches unity above 250 Å; values for α are not known at 10 Å. The actual thickness of the film of 10.8 Å may, therefore, be only three or four monolayers and conducting films of smaller thickness may not be feasible. The colour of the films obtained varied from light grey at 11 Å to greenish-grey as l approaches 50 Å.

We found that using gold as a nucleating layer did not produce ultra-thin, conducting films of platinum in this range.

It is known that the interatomic forces of attraction between the evaporant atoms, of gold or platinum in this case, cause liquid-like coalescence and consequent island formation, resulting in discontinuous films at thicknesses less than 50 Å. We postulate that, before the onset of island formation, a monolayer or so of a suitable nucleating material has the adatoms locked in the potential well sites on an ionic substrate surface. These then form an effective barrier against surface migration of the oncoming gold atoms and thus induce film growth in monolayers rather than discrete three-dimensional nuclei. The method may also be one with which to induce epitaxy. The choice of nucleating material seems critical.

Preliminary annealing studies on gold-on-platinum films 25 Å thick showed a slight decrease in R_μ up to 210° C; above this temperature R_μ increased slightly. The resistance of gold films alone increased irreversibly with temperature. Although detailed *in situ* electron microscopy and diffraction

studies¹² will be necessary to obtain direct evidence, the high electrical conductivity and annealing behaviour of films of gold on platinum may be taken as a reasonable indication that these are continuous. Further work on the ageing and annealing properties, together with a determination of the temperature coefficient of resistance of these films, is in progress. Other nucleating materials are also being investigated.

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Epoxy groups responsible for crosslinking in natural rubber

STORAGE hardening in natural rubber, which is of considerable industrial interest, has been ascribed to the crosslinking of carbonyl groups present in the rubber chain¹. Supporting evidence is that the crosslinking reaction can be prevented or accelerated by the addition of monofunctional or difunctional amines, respectively. These observations may be explained if the monofunctional amine is regarded as condensing with, and hence destroying, the carbonyl group, whereas the difunctional reagent is capable of reaction with carbonyl groups on separate chains, thus effecting a crosslink. The ability of dimedone (5,5-dimethylcyclohexane-1,3-dione) to inhibit the crosslinking reaction led to the conclusion that the groups were aldehydic¹.

Involvement of aldehyde groups in storage hardening necessitates the presence of complementary 'aldehyde condensing groups' (for example, amino groups) on the macromolecule². It is not easy to account for the presence of aldehyde groups, let alone 'aldehyde condensing groups', on the natural rubber molecule in terms of the known biosynthetic pathways for isoprenoid compounds.

Can other functional groups account for this behaviour? The presence of epoxy groups can provide a mechanism for storage hardening. Epoxidation of the olefinic double bond

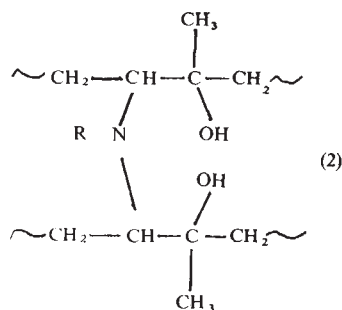
has ample precedents among naturally occurring compounds. All classes of terpenoid compounds (with the exception, hitherto, of natural rubber itself) include members that have epoxide groups, and they are found also on the isoprenyl residues of some other compounds of mixed biogenetic origin. Direct evidence for epoxide groups in natural rubber is presented later in this paper.

Storage hardening may be envisaged to occur by the following reactions:

(1) Epoxy groups are ring opened by naturally occurring amino-compounds in the latex, for example

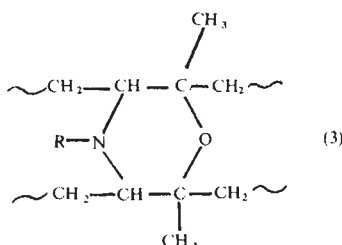


(2) This substituted amine could interact with an epoxy group on an adjacent chain with formation of a crosslink such as



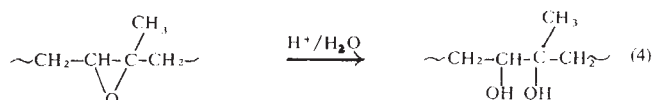
which may be partially formed at the latex stage.

(3) During storage and drying, ring closure of the product of reaction (2) could occur, forming an additional crosslink, for example, the cyclic structure



The presence of epoxy groups is fully consistent with the experimental results, which apparently favoured aldehydic groups, since ring opening of epoxy groups by amines is well known³. Similarly the reactions of epoxides with active hydrogens compounds analogous to dimedone have been reported⁴.

Furthermore the present proposals provide insight into the previously unexplained observations that the addition of sodium azide as a preservative to rubber latex leads to a decrease in the number of 'condensing groups'², and that conditions of low pH during coagulation of the latex reduces the extent of crosslinking on storage⁵. The former is readily explained since the azide ion cleaves epoxide rings to the β -azido alcohol⁶. Similarly the latter observation may be attributed to ring opening of epoxy groups under acidic conditions, for example



The resulting glycols have no crosslinking ability.

The presence of epoxy groups in natural rubber was demonstrated by making use of reaction (4). A fresh latex sample

was treated with acid in order to ring open the epoxide links. The resulting 1,2-diols were cleaved with lead tetraacetate by a method analogous to that used by Flory and Leutner⁷. The chain scission resulting from oxidation of the 1,2-diols was detected by viscometry. The intrinsic viscosity $[\eta]$ of a chloroform solution of the natural rubber sample, at 30°C, before and after treatment with lead tetraacetate is 3.75 and 0.39, respectively.

It is immediately apparent that considerable degradation has occurred. Unfortunately, the parameters of the Mark-Houwink viscosity equation for natural rubber in chloroform are not available but, if a reasonable value of $\alpha = 0.7$ is assumed, then the decrease in viscosity corresponds to a decrease in molecular weight by a factor of about 14. This in turn leads to an average number of about 13 epoxy groups per chain. The value is only approximate since not all of the epoxy groups may have ring opened but nevertheless it seems significant that the value is of the same order as that reported previously for 'aldehyde groups' in natural rubber⁸.

That the decrease in molecular weight, on degradation with lead tetraacetate, is due to the cleavage of 1,2-diols rather than oxidation of the double bonds is suggested by the rapid reaction, degradation being essentially complete within 30 min. Although oxidation of double bonds by lead tetraacetate has been reported, the reaction is slow and addition products rather than bond cleavage are observed⁹.

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BIOLOGICAL SCIENCES

Geographical uniformity of selectively neutral polymorphisms

IN a recent issue of *Nature*, Bulmer¹ criticised the 'neutral theory'² of protein polymorphisms on the ground that the infinite neutral allele hypothesis of Kimura and Crow³, when combined with Malécot's two-dimensional dispersal model^{4,5} leads to results which do not fit to actual data⁶⁻⁸. In this note, we intend to show that Bulmer's conclusions are entirely due to the unwarranted assumption of infinite population size inherent in Malécot's formulation, and that when the assumption is corrected, the data in fact are consistent with the neutral theory.

Bulmer claims that there are two contradictions. First, if we use Malécot's formulae for a continuous two-dimensional dispersal model, we get a rather strong geographical localisation of allele frequencies, whereas considerable uniformity is observed in the data. He uses Malécot's two quantities: the inbreeding coefficient (f_0) defined as the probability that

two homologous loci in an individual are identical by descent, and the coefficient of kinship (f_x) defined in a similar way, between two individuals at a distance x apart. Using Malécot's formula for f_0 and f_x which are valid only for a population distributed over a two-dimensional habitat of infinite size, Bulmer calculates the ratio, $\rho_x = f_x/f_0$. The numerical values of ρ_x presented in his examples certainly decreases quite rapidly as x increases. On the other hand, the estimates of this ratio using data from *Drosophila pseudoobscura* are very close to unity even for far distant localities, such as Texas, Colorado and California. He claims then that such a discrepancy cannot be resolved except under the absurd assumption that the root mean square dispersal distance, σ , is of the order of 2,000 km.

Secondly, he points out that the expected degree of homozygosity (f_0) would be much less than that of the data, unless the gene dispersion is very restricted. The average homozygosity found by Prakash *et al.*⁹ is 0.877. Bulmer claims, using Malécot's formula for f_0 , that to make f_0 to be about this magnitude, the mean square dispersal distance between the breeding places of parent and offspring (σ^2) must be an absurdly small value. For example, if the mutation rate u is 10^{-5} , then the number of flies within a circle of radius σ , that is $\pi\sigma^2\delta$, is about 0.38. This seems to be not only untrue but if the dispersion is restricted to this degree, ρ_x should decrease very rapidly.

It is important to realise that Malécot's formulae used by Bulmer are valid only for cases where the total population number (N) is much greater than the reciprocal of mutation rate (u) and the habitat is large. The size of habitat alone, however, is not sufficient, as long as the population is finite. Of course, if the population is truly infinite, his formulae are valid. On the other hand, if N and $1/u$ are about the same order of magnitude, it is necessary to use formulae valid for a finite population. For such a population, Malécot's formulae cannot be used even as an approximation, because they give entirely different values. Exact analysis for a finite case of torus-like space of size $L_1 \times L_2$ was obtained by Maruyama⁹. For a more realistic case of a rectangular habitat of size $L_1 \times L_2$, although exact solutions were not obtained, it turned out that they can be approximated by solutions of the torus-like space of size $2L_1 \times 2L_2$ (ref. 9). So, we use the torus-like space in the following discussion. If we designate position in the space by Cartesian coordinate system and let $f(x, y)$ be the probability that two homologous genes separated distance x and y along the first and second axes respectively are identical by descent, then

$$f(x, y) = \frac{(1-u)^2(1-f_0)}{2\delta L_1 L_2} \sum_{m=0}^{\infty} \sum_{n=0}^{\infty} \frac{\Delta_m \Delta_n R_{mn}}{[1 - (1-u)^2 R_{mn}]} \cos \frac{2\pi mx}{L_1} \cos \frac{2\pi ny}{L_2} \quad (1)$$

where $\Delta_0 = 1$ and $\Delta_k = 2$ if $k \neq 0$, δ is the population density and

$$R_{mn} = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} r(\xi, \eta) \cos \frac{2\pi m\xi}{L_1} \cos \frac{2\pi n\eta}{L_2} d\xi d\eta$$

in which $r(x, y)$ is the probability that a zygote is formed between gametes of parents born distance $\sqrt{(x^2 + y^2)}$ apart. As a special case, if $r(x, y)$ is a two-dimensional normal density and the migration is isotropic,

$$r(x, y) = \frac{1}{2\pi\sigma^2} \exp [-(x^2 + y^2)/(2\sigma^2)], \quad (2)$$

and

$$R_{mn} = \exp [-2\pi^2\sigma^2(m^2/L_1^2 + n^2/L_2^2)]$$

TABLE 1. Theoretical values of the inbreeding coefficient and genetic correlation

$4Nu$	N	u	$\sigma^2\delta$	f_0	Furthest distance possible/ σ	$f_{\min}^*/f_0$
0.08	2×10^6	10^{-8}	2	0.9283	707	0.9624
0.12	3×10^6	10^{-8}	3	0.9862	707	0.9624
0.12	3×10^6	10^{-8}	1.5	0.8998	1000	0.9235
0.2	2.5×10^7	2×10^{-9}	100	0.8335	354	0.9983
0.2	2.5×10^7	2×10^{-9}	50	0.8337	500	0.9966
0.4	10^7	10^{-8}	10	0.7206	707	0.9667
0.4	10^7	10^{-8}	5	0.7268	1000	0.9351
1.0	2.5×10^6	10^{-7}	10	0.5196	354	0.9194
4.0	10^8	10^{-8}	100	0.2051	707	0.9664
10.0	2.5×10^7	10^{-7}	100	0.0976	354	0.9191
40.0	10^8	10^{-7}	50	0.0395	1000	0.5823
40.0	10^7	10^{-6}	5	0.1674	1000	0.0818
100.0	2.5×10^7	10^{-6}	50	0.0263	500	0.3308

* $f_{\min}$ is the kinship coefficient between two individuals furthest apart.

In our formula (1), f_0 is involved implicitly, but it can be determined explicitly,

$$f_0 = \frac{(1-u)^2 S}{2\delta L_1 L_2 + (1-u)^2 S} \quad (3)$$

where

$$S = \sum_{m=0}^{\infty} \sum_{n=0}^{\infty} \frac{\Delta_m \Delta_n R_{mn}}{[1 - (1-u)^2 R_{mn}]}$$

Using formulae (1) and (3) with $r(x, y)$ given by (2), and assuming $L_1 = L_2$, we have carried out extensive numerical calculations using our computer TOSBAC 3400. Contrary to Bulmer's claim, we found that theoretical expectations and data are consistent. If Nu is 1 or less and $\sigma^2\delta$ is of order of 100 or 10 or even less, ratio $f(x, y)/f_0$ stays very close to 1 and it is roughly independent of distance and habitat size. Here, a remark on mutation rate is in order. As pointed out by Kimura and Ohta², the appropriate mutation rate for neutral alleles is at most 10^{-7} per yr per locus. So, for *Drosophila* whose generation time is some 2 weeks, we should take $u = 10^{-8}$ or less to be consistent with the neutral theory. From these assumptions, it also follows that if we take $4Nu = 0.1 \sim 0.2$, the value of f_0 turns out to be about right order of magnitude consistent with the neutral theory². Note here that N represents the effective population size rather than the actual size. Numerical examples are presented in Table 1, where $f_{\min}$ is the kinship coefficient between two individuals furthest distance apart. It should be emphasised that the finiteness of population makes the situation completely different from that of a truly infinite case. Thus, Bulmer is unwarranted in using Malécot's formulae which led him to the erroneous conclusions which seem to be inconsistent with data: Bulmer's dilemma is entirely due to an incorrect assumption of infinite population, but this can be simply resolved by the correct assumption. This may be evident by comparing values in column $f_{\min}/f_0$ of Table 1 in this paper with his tables.

It seems appropriate to discuss here the influence of the dimensionality of space on these properties. Through numerical calculations, we found that a two-dimensional population tends to be nearly panmictic, particularly if $4Nu$ is order of 1 or less, and that panmixity can be attained easily. As a consequence, f_0 is related to N almost directly. On the other hand, in an one-dimensional (or a long but narrow) population, the situation is quite different, and the local differentiation appears, if $\sigma^2\delta$ is small compared with the habitat length. When the habitat size is sufficiently long, the value of f_0 becomes nearly independent of the total popula-

tion size. In such a circumstance, a formula derived assuming an infinite population size gives approximately correct values for f_0 and f_x . But as long as the value of f_0 is influenced by the total size, which is often the case in a two-dimensional population, the finiteness must be taken into account. This is where Bulmer has made an error. We conclude that the pattern of geographical distribution of protein polymorphisms in *Drosophila* as discussed by him is indeed consistent with the neutral theory.

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Enzymatic acylation of histidine to mengovirus RNA

PLANT virus RNA from the TYMV family¹⁻³, TMV^{4,5} and BMV⁶ can be enzymatically esterified with valine, histidine and tyrosine, respectively. The aminoacylation of intact or fragmented plant virus RNA⁷, may be an important biological function and, therefore, the question as to whether some mammalian viral RNA can be charged with amino acids becomes significant. In this work, we show that mengovirus RNA can, in fact, be specifically acylated with histidine by a mouse liver aminoacyl-tRNA synthetase preparation.

Mengovirus was grown in mouse L-929 cells (clone CCL1) and precipitated by polyethylene glycol. The virus particles were purified by banding in CsCl before the extraction of viral RNA (see legend to Fig. 1). This step was found to be important for the elimination of any contaminating cellular tRNA. To test the homogeneity of the viral RNA, samples were analysed by electrophoresis on polyacrylamide-agarose gels^{8,9}. No detectable amounts of RNA of low molecular weight could be found in the viral RNA preparation. The apparent molecular weight of the mengovirus RNA was estimated as 2.3×10^6 , by comparing its migration with that of TMV RNA and total *E. coli* RNA (Fig. 1). Our value for the molecular weight differs slightly from previous values for mengovirus, which were based on hydrodynamic measurements (1.74×10^6 , ref. 10) or sucrose density gradient sedimentation analyses (2.8×10^6 , ref. 11).

Mengovirus RNA can be acylated with a mixture of 16 amino acids by mouse liver aminoacyl-tRNA synthetase (Table 1). On the other hand, MS2 bacteriophage RNA or mouse liver rRNA are not aminoacylated under the same experimental conditions. It is interesting that the heterologous aminoacyl-tRNA synthetase from *E. coli* failed to acylate mengovirus RNA, MS2 bacteriophage RNA or TMV RNA. Mengovirus RNA was found to be esterified solely with histidine by the mouse liver aminoacyl-tRNA synthetase preparation. This was deduced from the finding that the acylation of mengovirus RNA with a mixture of labelled

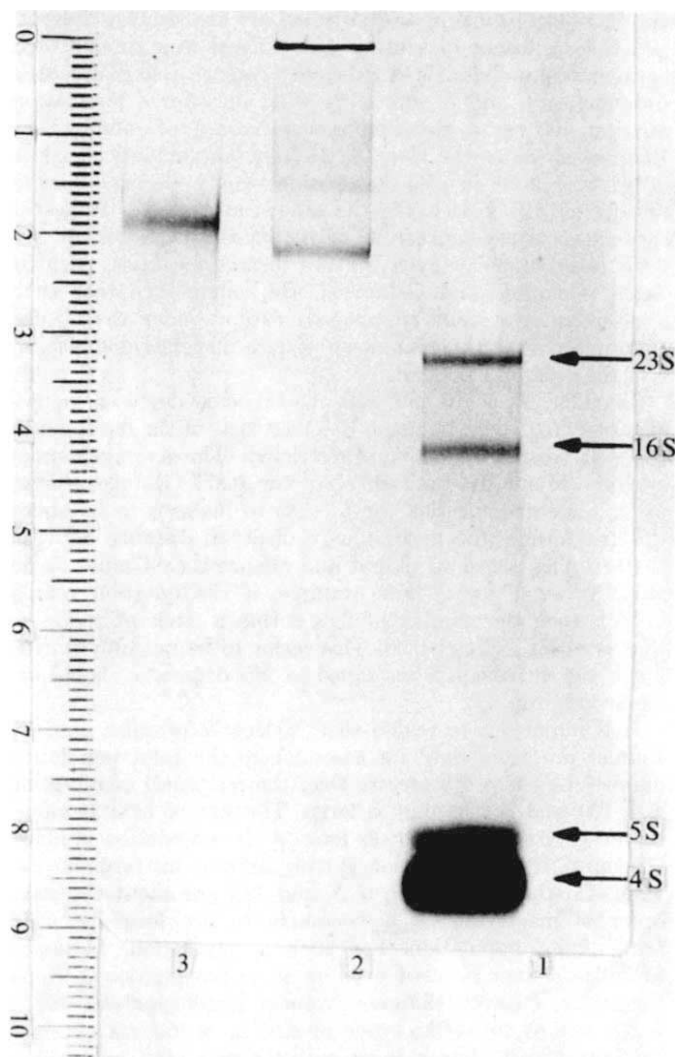


FIG. 1 Polyacrylamide-agarose gel electrophoretic patterns of mengovirus RNA, TMV RNA and total *E. coli* RNA. Confluent monolayers of mouse L-929 cells (clone CCL1) were infected with 2 PFU per cell of mengovirus. After incubation for 1 h at 37° C, 40 ml of Eagle's MEM containing 8% calf serum was added to each 200 cm² bottle. Incubation was continued for 18 h, at which time the cell mass was dislodged by gentle shaking. The suspension was frozen and thawed and the lysate was freed of cell debris by centrifuging at 1000g for 5 min at 4° C. The mengovirus suspension was supplemented with 6% polyethylene glycol (6000, Union Carbide) and 0.01% β -mercaptoethanol and stirred at 0° C for 1 h. The precipitate was collected by centrifugation at 12,000g for 20 min and suspended with the aid of a glass-teflon homogeniser in 100 mM Tris-HCl, pH 7.9 and 10 mM EDTA¹¹. The density of the suspension was adjusted to 1.332 g cm⁻³ with CsCl and the virus particles were banded by centrifugation at 40,000 r.p.m. in a type 40 fixed angle rotor in a Beckman Model L ultracentrifuge for 18 h. The band containing the virus particles was collected, diluted three-fold with water and centrifuged for 2 h at 40,000 r.p.m. The RNA was isolated from the virus suspension by phenol-chloroform extraction, followed by ethanol precipitation¹⁵. Each RNA (1.0–5.0 μ g) sample was applied to an 1.7% polyacrylamide–0.5% agarose slab gel. Electrophoresis was carried out for 2.5 h at 0° C at 11.4 V cm⁻¹. The gels were stained with Stains all[®]. Slot 1, Total *E. coli* RNA; slot 2, TMV RNA; slot 3, mengovirus RNA.

amino acids is hardly suppressed by the addition of excess non-radioactive mixture of amino acids lacking histidine, whereas the addition of excess non-radioactive histidine completely inhibited the reaction.

We have devised an electrophoretic method to determine the size of individually labelled aminoacylated tRNA species.

To preserve the aminoacyl-RNA ester bond, the aminoacyl-RNA was acylated with N-hydroxysuccinimide acetyl ester to yield N-acetylaminoacyl-RNA. We synthesised the latter, since N-substituted aminoacyl-tRNA derivatives have been shown to be considerably more resistant to hydrolysis than the corresponding unblocked aminoacyl-tRNA derivatives (compare ref. 12). Using N-acetyl- ^3H -aminoacyl-tRNA molecules, we have been able to estimate their size by monitoring the radioactivity of the sliced gel. Under these conditions, unblocked aminoacyl-tRNA molecules are completely hydrolysed. Figure 2a shows the electrophoretic migration of mouse liver N-acetyl- ^3H -histidyl-tRNA. The electrophoretic pattern of mengovirus RNA bound to N-acetyl- ^3H -histidine,

TABLE 1 Aminoacylation of viral RNAs

Source of RNA	Amino acids	Amino acids incorporated (c.p.m. per 10 μg RNA)	
		Mouse liver synthetase	<i>E. coli</i> synthetase
Mengovirus	^3H -mixture	2,000	170
TMV	^3H -mixture	4,800	270
MS2 bacteriophage	^3H -mixture	40	190
Mouse liver rRNA	^3H -mixture	80	—
Mengovirus	^3H -mixture + unlabelled 15 amino acid mixture lacking histidine	1,900	—
Mengovirus	^3H -mixture + unlabelled histidine	60	—
Mengovirus	^3H -histidine	3,600	—
TMV	^3H -histidine	7,800	—

The incubation mixture (0.05 ml) contained: 20 mM Tris-HCl, pH 7.5, 1 mM ATP, 10 mM MgCl_2 , 3 mM dithiothreitol, 0.5 μCi of a mixture of 16 ^3H -labelled amino acids (^3H -reconstituted protein hydrolysate, Schwartz/Mann, consisting of alanine, arginine, cystine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine) or ^3H -histidine (58 Ci mmol $^{-1}$ Amersham), 10–20 μg RNA and 50 μg mouse liver aminoacyl-tRNA synthetase, or 5 μg of partially purified mouse liver histidyl-tRNA synthetase or 5–10 μg of an *E. coli* enzyme preparation. Where indicated, a mixture of 0.08 mM each of 15 unlabelled amino acids or 0.08 mM histidine was added. The incubations were carried out at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of bovine serum albumin solution (0.25 mg) followed by precipitation with cold 5% trichloroacetic acid. The precipitate was filtered through glass filters (Whatman GF/C) washed and counted. The mouse liver aminoacyl-tRNA synthetase was partially purified from a 105,000g supernatant by phase partition 13 . The mouse liver histidyl-tRNA synthetase was further purified by DEAE-cellulose chromatography. The *E. coli* aminoacyl-tRNA synthetase was prepared according to Muench and Berg 14 .

and run in parallel on the same gel, is shown in Fig. 2b. The size of the major RNA peak bearing ^3H -histidine, was estimated to be between 0.20×10^6 to 0.35×10^6 daltons. In addition, a small part of the ^3H -histidine was attached to RNA of molecular weight 1.25×10^6 . When the mengovirus N-acetyl- ^3H -histidyl-RNA preparation was treated with pancreatic RNase and then analysed by gel electrophoresis, no radioactivity could be detected in the various gel slices.

From these results it is clear that the mengovirus RNA is enzymatically acylated with histidine. It seems that mengovirus RNA is cleaved during the aminoacylation reaction, probably because of contamination of the crude mouse liver aminoacyl-tRNA synthetase preparation with traces of endo-

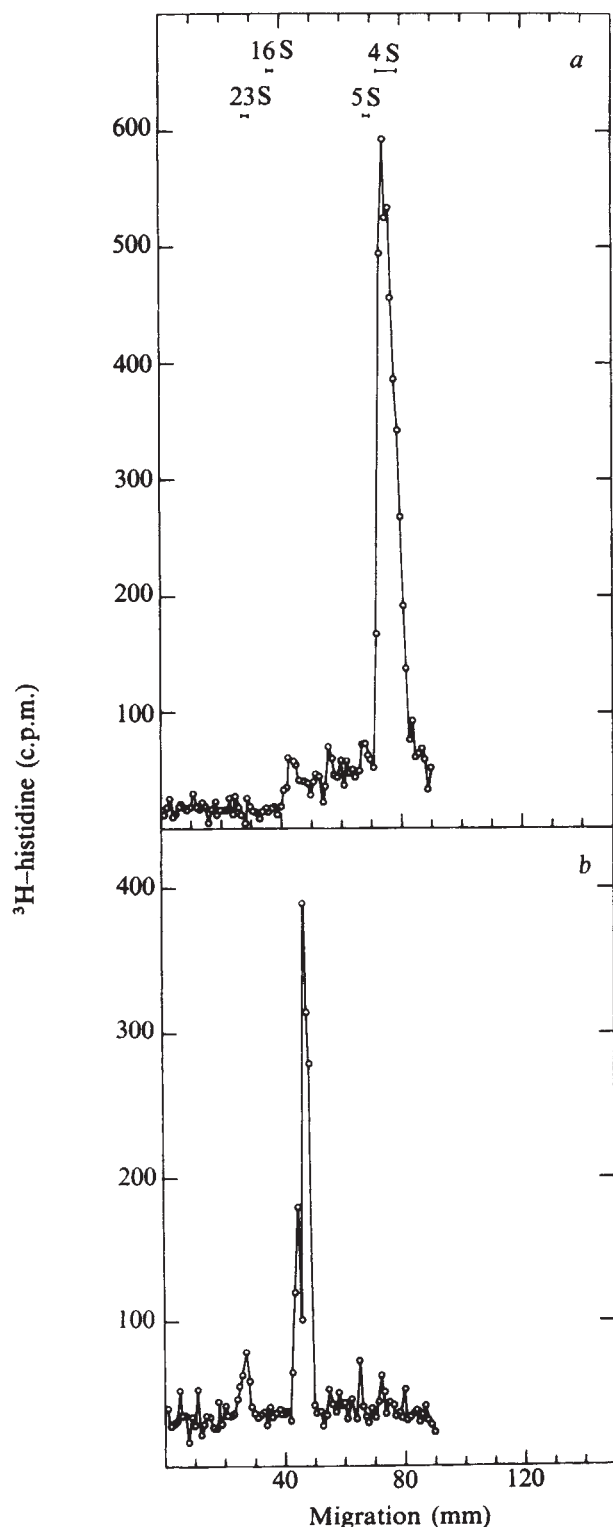


FIG. 2 Polyacrylamide-agarose gel electrophoresis of mouse liver N-acetyl- ^3H -histidyl-tRNA (a) and mengovirus N-acetyl- ^3H -histidyl-RNA (b). Mouse liver tRNA and mengovirus RNA were acylated with ^3H -histidine (58 Ci mmol $^{-1}$) with mouse liver histidyl-tRNA synthetase, as described in the legend to Table 1. To 0.05 ml of the labelled aminoacyl-RNA solution, 0.025 ml of 1 M sodium acetate, buffer, pH 4.2 and 0.15 ml of ethanol were added. The precipitated RNA was dissolved in 0.025 ml of 0.01 M sodium acetate buffer, pH 5.0 and a solution of 0.05 ml of dimethylsulphoxide containing 5 mg of N-hydroxysuccinimide acetyl ester was added. The mixture was then treated as previously described 12 . The mouse liver N-acetyl- ^3H -histidyl-tRNA (663 c.p.m. μg^{-1}) and the mengovirus N-acetyl- ^3H -histidyl-RNA (179 c.p.m. μg^{-1}) preparations were applied to two different slots on a 1.7% polyacrylamide-0.5% agarose slab gel. In a third slot 5 μg of total *E. coli* RNA was added. Electrophoresis was carried out for 2 h at 0°C at 11.4 V cm $^{-1}$. The gel was stained with Stains all and then sliced into 1 mm fractions. Each slice was transferred to a 3.5 ml vial, incubated with 0.3 ml NCS (Amersham/Searle) for 30 min at 20°C and then 3 ml of toluene-based scintillation fluid was added. The vials were inserted into standard 20 ml scintillation vials and counted in a Packard Tricarb scintillation counter.

nucleases. It is still not clear whether specific cleavage of mengovirus RNA is required for its histidine-acceptance activity. The aminoacylated mengovirus RNA fragments have, however, a much larger molecular weight than that of tRNA and, thus, the possibility of tRNA contamination can be excluded. Moreover, the reaction of mengovirus histidyl-RNA with N-hydroxysuccinimide acetyl ester was carried out in 66% dimethylsulphoxide. Under these conditions any contaminating non-covalently linked tRNA would have been dissociated from the mengovirus RNA.

To estimate the extent of mengovirus acylation, we have increased the histidine concentration in the acylation mixture by using ^{14}C -labelled histidine (Schwartz/Mann 342 mCi mmol $^{-1}$). Under these conditions, 0.18 mol histidine are acylated per mol of mengovirus RNA, as compared with a value of 0.30 mol obtained with TMV RNA. The latter value confirms the results of Litvak *et al.*⁵.

In this study we have shown that specific aminoacylation of viral RNA is not confined only to plant viruses. It will be interesting to determine whether other mammalian viral RNA preparations also serve as acceptors for amino acids. In the case of poliovirus RNA, no aminoacylation could be detected by Öberg and Philipson⁴. Whether this is due to the nature of poliovirus or to differences in the aminoacylation system remains to be determined.

The biological function of the amino acid acceptor activity of plant virus and mengovirus RNA is not known. It is possible that the aminoacylation of these viral RNA species may have some regulatory function in protein synthesis or that aminoacyl-RNA may be involved in the recognition site of viral RNA replicase.

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Structure of inositol hexaphosphate-human deoxyhaemoglobin complex

D-2,3-DIPHOSPHOGLYCERATE (DPG) facilitates the transfer of oxygen from human red blood cells to the tissues by lowering the oxygen affinity of haemoglobin¹. It does so by combining preferentially with one of the two alternative forms of haemoglobin, namely the deoxy form, in the ratio of 1 mol per mol tetramer. Its binding site was predicted from biochemical and model building experiments and then determined directly by X-ray crystallography². The site lies at the entrance to the central cavity between the N-termini of the β chains and is surrounded by four pairs of basic groups: the α amino group of valine 1 and the side chains of histidines 2 and 143, and of lysine 82. The basic groups are related in pairs by the molecular dyad and arranged so as to complement the acidic groups of DPG by forming seven salt bridges (Fig. 1). On oxygenation the N-termini of the β chains move apart and the cavity closes up so that the stereochemical complementarity is lost³. Oxyhaemoglobin does also bind DPG, but much more weakly, and the binding site is still unknown.

Several other multivalent anions, including inositol hexaphosphate (IHP), ATP, GTP and $[\text{Fe}(\text{CN})_6]^{4-}$, also lower the oxygen affinity of haemoglobin. The most effective and biologically also the most important of them is IHP, as it is closely related to the inositol pentaphosphate which takes the place of DPG in regulating the oxygen affinity of bird erythrocytes^{4,5}. We have determined the binding site of IHP by X-ray analysis and model building. Its six phosphate groups are coordinated to the same basic groups of the β chains as the single carboxyl and the two phosphates of DPG, and the binding of the two compounds also produces similar changes in the tertiary structure of the β subunits.

Crystals of human deoxyhaemoglobin were prepared by the method of Perutz⁶, except that the ammonium phosphate normally added to the more than 2 M ammonium sulphate solution was replaced by 20 mM IHP at pH 6.5. At this high ionic strength, however, IHP failed to bind. The crystals were therefore soaked for 1 d in an aqueous solution of 70% (v/v) 2-methyl-2,4-pentanediol (MPD) + 1 mM IHP + 20 mM bis Tris adjusted to pH 6.5 with HCl. Other crystals were grown in the standard way (that is, without IHP) and soaked in a solution of 70% MPD + 20 mM bis Tris at pH 6.5. The structure of the haemoglobin molecules in such crystals is the same as that in crystals suspended in concentrated ammonium sulphate². The intensities of about 14,000 reflections within a limiting sphere of 3.5 Å $^{-1}$ from crystals in MPD with and without IHP were measured on a Crystallog four-cycle diffractometer. A difference Fourier synthesis was calculated using $(|F_{+IHP}| - |F_{-IHP}|)$ as the coefficients to-

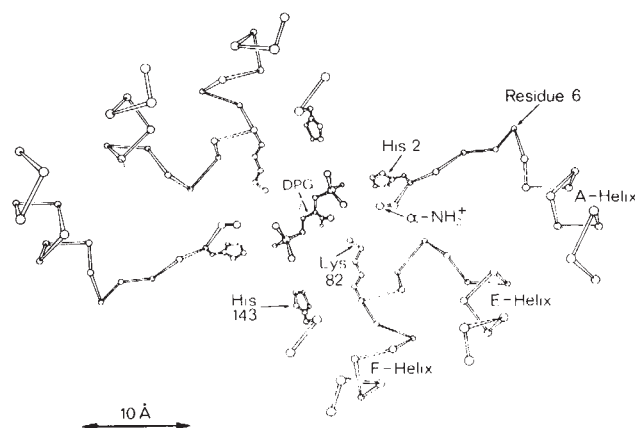


Fig. 1 Binding of DPG to human deoxyhaemoglobin.

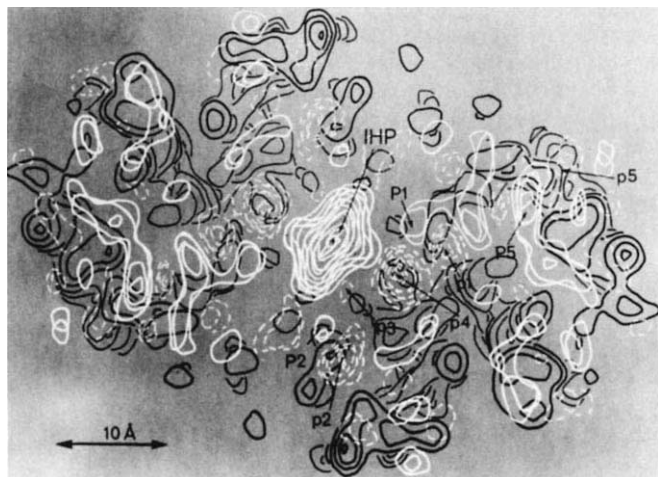


FIG. 2 Fourier map showing the binding of IHP. Black contours: sections -15 to -23 Å of the electron density map of human deoxyhaemoglobin which includes the same parts of the molecule, in the same orientation, as the model sketched in Fig. 1. Difference peaks P1 and p1 straddle the N terminus, peaks P5 and p5 the A helix, peaks P2 and p2 histidine 143. The positions of helices E and F and of the EF corner are obvious by reference to Fig. 1. White contours: difference electron density map $\pm$ IHP. Solid contours and upper case letters mark positive difference density; broken contours and lower case letters, negative difference density.

gether with the phase angles determined by Muirhead and Greer⁷.

The difference electron density map is dominated by a massive and markedly asymmetrical positive peak at the entrance to the central cavity between the N termini of the β chains. The asymmetry may be an artefact, because the dyad axis passing through the centre of the haemoglobin molecule is not a crystallographic one, so that errors in intensity measurement and in phasing have different effects on points in the electron density which are related by the molecular dyad, but are not crystallographically equivalent. The difference map was therefore averaged about the molecular dyad, as in the previous study of DPG, and then sampled in planes 1 Å apart perpendicular to that axis.

Figure 2 shows a projection of several sections of the symmetry-averaged map. The large difference peak is cross shaped and has its maximum at a level 12 Å below that of the iron atoms in the β subunits. To find the orientation of the IHP molecule, a model built according to the structure of its sodium salt⁸ was viewed against the difference map in same Richards box⁹ in which a model of human deoxyhaemoglobin had been constructed (A. A., L. F. Ten Eyck, and G. Fermi, unpublished). The model of IHP fitted the difference map only with the cyclohexane ring parallel, but not perpendicular, to the dyad, and it fitted well with the line joining phosphorus atoms 3 and 6 normal to the dyad and inclined at an angle of 32° to x (Fig. 3). An equally good fit can be obtained after rotating the model by 120° so that phosphate 1 occupies the place of 3, and 4 that of 6. The distance between the phosphorus atoms of either of these two phosphates is 7.5 Å as compared to 6.5 Å between those of fully stretched DPG (Fig. 1). In either of these orientations our very limited resolution allows some latitude for rotation of the individual phosphate groups about both the C-O and the O-P bonds and therefore the fit should not be regarded as strong evidence in favour of the conformation of IHP in deoxyhaemoglobin being exactly the same as in the crystals of its sodium salt.

Phosphates 3 and 6 fit in well between the two α amino groups, which are 4 Å from the nearest phosphate oxygen in the undistorted deoxy structure (Fig. 4). This distance is too large for salt bridges, so that IHP (and DPG) pull the

two α amino groups closer together. The evidence for this comes from a pair of positive (P1) and negative (p1) peaks which straddle the native electron density peaks of valines 1 β , showing that the N termini move towards the phosphates; and peaks P5 and p5 which show that the entire A helix moves towards the centre of the molecule. Peaks P2 and p2 show that histidines 143 β also move towards the phosphates. As in the DPG map, a large negative peak (P4) overlaps a peak in the native density, indicating that the atoms represented by that peak are removed by IHP. The peak lies outside the density representing the protein and is probably due to an inorganic anion, either sulphate or phosphate, bound to the α amino groups in the absence of IHP. Unlike the DPG map, the IHP map shows hardly any movement of lysine

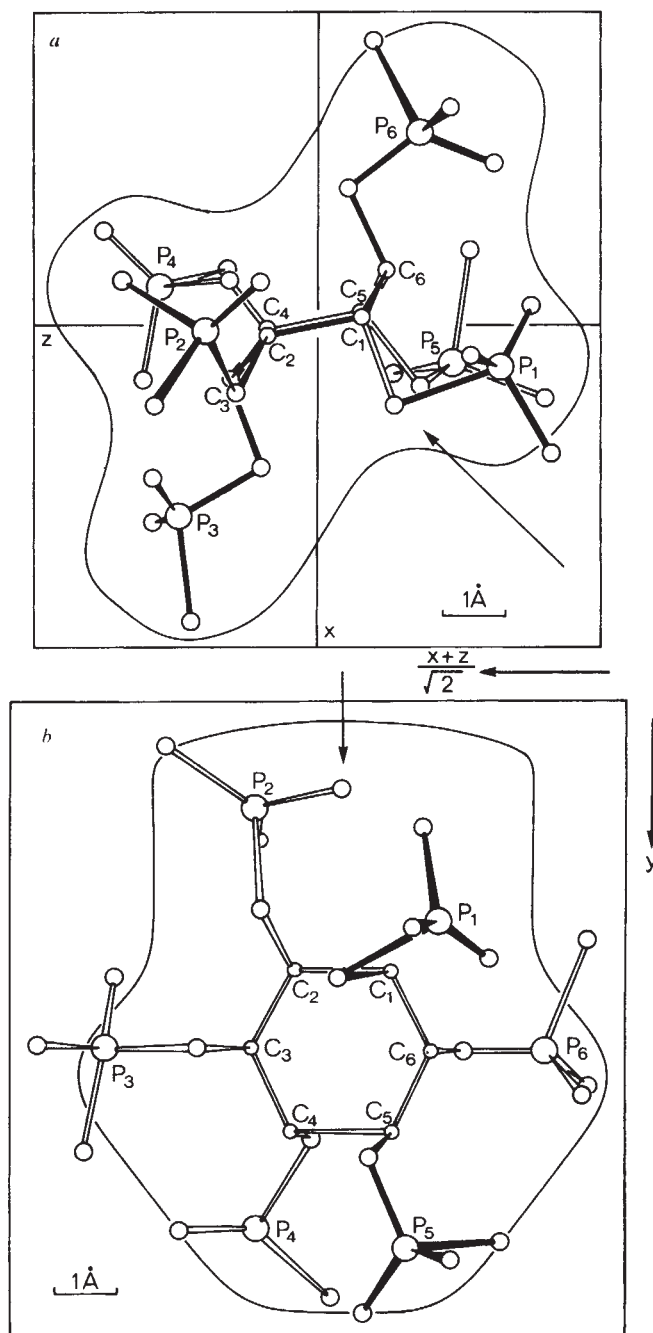


FIG. 3 Model of IHP fitted to the difference map. *a*, In projection on a plane normal to the molecular dyad superimposed on the outline of the central section ($y = -19$) through the difference peak. *b*, In projection along the diagonal between x and z against the outline of the central section through the difference peak. The arrow in (*b*) shows the direction of view in (*a*) and vice versa.

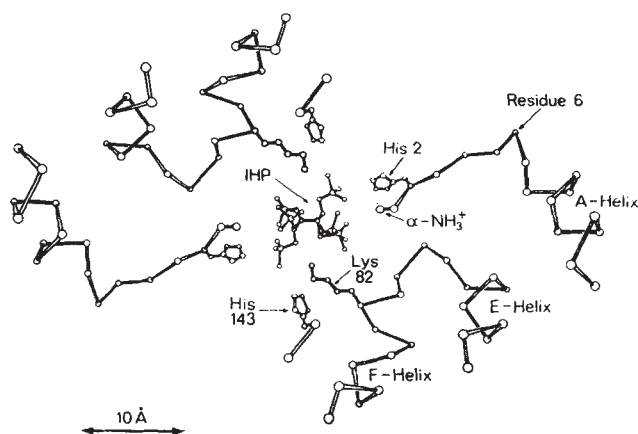


FIG. 4 Binding of IHP to human deoxyhaemoglobin. Note that the conformation of lysine 82 is different from that in Fig. 1. The side chain of asparagine 139 which could form hydrogen bonds with phosphates 1 and 2, lies approximately above that of lysine 82.

82 or of histidine 2. Lack of the former is explained by the fact that the side chain lies in the correct position to form salt bridges with phosphates without having to move. The reason for the absence of difference peaks near histidine 2 is not clear because this residue must move with the N-terminus and helix A. The amino groups of asparagines 139 β are in positions that allow them to form hydrogen bonds with phosphates 1 and 2.

The DPG difference map showed widespread disturbances to the tertiary structure of the β subunits, and the same are seen in the IHP complex. The movement of the A helix has already been referred to. Weaker difference peaks along helices E and F, and along helix H (not shown in Fig. 2) point to structural disturbances that radiate from the IHP binding site and extend as far as the haem. At our present resolution of 3.5 Å the exact nature and magnitude of these disturbances cannot be determined, but they may be such as to oppose the transition of the tertiary structure from the deoxy to the oxy form, in which case both DPG and IHP should decrease the oxygen affinity of the β subunits in the quaternary deoxy structure.

In summary, the mode of binding of IHP to human deoxyhaemoglobin is similar to that of DPG. There are the same eight basic groups to form salt bridges with the six phosphates of IHP as with the two phosphates and one carboxyl of DPG, but in addition asparagines 139 may form hydrogen bonds with two of the phosphates. Titration of IHP shows that it carries about eight negative charges at pH 7.3. Two of the basic groups of the globin, the ϵ amino groups of the lysines, have pK 's of 10.5 and will therefore be fully charged in the absence of IHP. The four imidazoles and two α amino groups have pK 's in the neutral range which will rise on combination with IHP, while those of the phosphates will fall. The net effect on the bases, however, clearly dominates, because combination of deoxyhaemoglobin with IHP is accompanied by an uptake of protons¹⁰. On being bound to crystalline deoxyhaemoglobin grown in concentrated buffers both DPG and IHP displace one inorganic anion from each of the β chains. They both create perturbations to the tertiary structure of the β subunits that extend to the haems and could lower the oxygen affinity by stabilising the quaternary deoxy structure of the tetramer and by constraining the tertiary structure of the β subunits.

In avian erythrocytes inositol 1,3,4,5,6-pentaphosphate (IPP) takes the place of DPG as a regulator of oxygen affinity⁵. The only avian haemoglobin sequence determined so far is that of the chicken¹¹. If, as seems likely, IPP takes up the same position in chicken deoxyhaemoglobin as IHP does in human, the number of basic groups facing it would

be 12 instead of eight, and would include Val 1, His 2, Lys 82, Arg 135, His 139 and Arg 143. Therefore, the binding energy should be very high indeed.

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β -2-Microglobulin is part of the HL-A molecule in the lymphocyte membrane

It has been shown that papain-solubilised HL-A antigens consist of two polypeptide chains, which may be separated under dissociating conditions^{1,2}. Peterson *et al.*³ have shown that β -2-microglobulin (β -2-m) constitutes one of the two polypeptide chains in papain-solubilised HL-A antigens. In these antigens, β -2-m represents a constant small subunit (molecular weight 11,800), whereas a larger subunit (30,000–31,000) carries the allo-antigenic specificity. The studies by Peterson *et al.* do not exclude the possibility that the two subunit structure of HL-A antigens might be a product of the solubilisation process. It has previously been reported⁴ that β -2-m, which also occurs in biological fluids⁵, is present in high concentrations on the surface of lymphocytes. The

First treatment		Second treatment	
R-anti- β -2-m, FITC-anti-Rlg		Anti-HL-A TRITC-anti-Hlg	
Anti-HL-A, TRITC-anti-Hlg		R-anti-R-2-m FITC-anti-Rlg	
R-ALS FITC-anti-Rlg		Anti-HL-A, TRITC-anti-Hlg	

β -2-m ● HL-A ○ Species-specific determinants(ALS) ≡

FIG. 1 Diagram of cell membrane antigen aggregation. Lymphocytes were first incubated with one set of antibodies at room temperature followed by incubation for 30 min at 37° C to induce capping of a cell membrane antigen. The cells were then reincubated with other antibodies at 4° C to determine whether the antigenic determinants detected by the second antibody co-capped with the antigen detected by the first antibody. 1, R-anti- β -2-m: Rabbit anti-human- β -2-microglobulin; 2, R-ALS: Rabbit anti-human lymphocyte serum; 3, Anti-HL-A: Pooled multi-specific anti-HL-A; 4, FITC-anti-Rlg: Fluorescein-labelled swine anti-rabbit-immunoglobulin; 5, TRITC-anti-Hlg: Rhodamine-labelled-goat anti-human-immunoglobulin.

TABLE 1 Aggregation/blocking of β -2-m, HL-A or species-specific antigens on human lymphocytes

Pretreatment*	Background (AB-serum)	Microcytotoxicity test results	
		Anti-HL-A (specific for the cells)	Anti- β -2-m (undiluted)
None (native cells)	-†	+++	+++
Anti- β -2-m	(+)	(+)	(+)
Anti- β -2-m + β -2-m	(+)	+++	+++
Anti-HL-A (pool of multispecific sera)	(+)	(+)	+++
Anti-lymphocyte serum (ALS) (1:64)	(+)	+++	+++
			(+)

* Sensitised for 2 h at 20° C, washed, incubated with anti-immunoglobulin for 30 min at 4° C, washed, and incubated 30 min at 37° C.

† Strength of the reactions: - = < 5% lysis; (+) = 5%-20%; ++ = 20%-40%; +++ = 20%-60%; ++++ = > 60%.

present study was designed to establish whether β -2-m is part of the intact HL-A molecule in the cell membrane. Experiments were performed in which the blocking of HL-A antigens^{6,7}, β -2-m and other lymphocyte membrane components were induced by selected antisera. Furthermore, to study the relationship between these cell membrane components and the antigen receptors on lymphocytes, the different antisera were also tested for their ability to induce or inhibit lymphocyte activation *in vitro*. Methodological details are published elsewhere⁸.

Anti-human- β -2-m (Dacopatts, 2900 Hellerup, Denmark, Catalogue Code T-072 Lot 023) was prepared in rabbits by immunisation with highly purified β -2-m from urine; the antibody preparation was immunochemically monospecific for β -2-m. Aggregation/blocking of cell membrane antigens was carried out by indirect technique. In 0.2 ml Hanks BSS (Flow Laboratories, Irvine, Scotland), 400,000 human lymphocytes prepared by Ficoll/Isopaque flotation, were first incubated with 0.2 ml of either undiluted anti- β -2-m, two rabbit anti-human lymphocyte sera⁹ (ALS) diluted 1:64, or with undiluted multispecific cytotoxic anti-HL-A serum. Preliminary experiments showed that these antiserum concentrations gave optimal cap formation. To potentiate aggregation/capping, the cells were then incubated with 0.2 ml anti-immunoglobulin (FITC-labelled or unlabelled swine anti-rabbit immunoglobulin (Dacopatts 21090 or F2190) or rhodamine-labelled goat anti-human immunoglobulin (Highland, Los Angeles) and warmed to 37° C for 30 min^{6,7}.

By microcytotoxicity testing¹⁰, undiluted anti- β -2-m induced 60-90% lysis of all human lymphocytes tested; the lysis was reduced to 30-40% after dilution 1:2, and lost upon dilution above 1:8. When the lymphocytes were first incubated with anti- β -2-m and anti-rabbit immunoglobulin, immediately followed by microcytotoxicity typing¹⁰, all detectable HL-A antigens on the tested cells were completely blocked; that is, none of the antigens belonging to three segregant SD series⁷ could be detected (Table 1). After incubation in a 1:1 mixture of Medium 199 (Flow Laboratories) and AB serum for 24 h at 37° C, blocked cells again showed a normal typing pattern. Preincubation of the cells with pooled anti-HL-A sera blocked the expression of HL-A antigens, but did not prevent the cells from being lysed by anti- β -2-m (Table 1), thus indicating an excess of β -2-m compared with HL-A in the cell membrane.

Aggregation of cell membrane antigens was also studied by immunofluorescence of double-stained cells. Aggregation was induced during the first staining procedure by warming to 37° C for 30 min; and the second staining step was carried out at 4° C to avoid additional aggregation of membrane components. When cells were first incubated with anti- β -2-m and stained with FITC-labelled anti-rabbit immunoglobulin, followed by incubation with anti-HL-A serum and staining with rhodamine-labelled anti-human immunoglobulin, the red fluorescence of the HL-A antigens was superimposed on the green fluorescence of β -2-m, which showed aggregation into patches and caps in virtually all cells. Figure 1 shows these results in diagrammatic form. (The cells were examined immediately with a Leitz Ortholux microscope equipped with

a Ploem-type vertical illuminator, and care was taken to keep the preparations cold). In experiments where the cells, after incubation with anti- β -2-m and anti-immunoglobulin were incubated with ALS and fluorescein-labelled anti-rabbit immunoglobulin, marked diffuse fluorescence of the whole cell membrane could be observed.

Thus both microcytotoxicity tests and immunofluorescence demonstrate that anti- β -2-m concomitantly aggregated/blocked β -2-m and HL-A molecules in the cell membrane. This strongly indicates that β -2-m is part of the native HL-A antigens in the cell membrane. By contrast, anti- β -2-m did not coaggregate antigens against which our ALS were directed.

When lymphocytes were preincubated with pooled anti-HL-A sera followed by rhodamine-labelled anti-human immunoglobulin, and then reincubated with anti- β -2-m and double-stained with FITC-labelled anti-rabbit immunoglobulin, immunofluorescence microscopy showed green stain superimposed on red patches and caps, but also diffusely distributed over the rest of the cell membrane, confirming the observations by cytotoxicity tests that there is an excess of β -2-m compared with HL-A antigens in the cell membrane.

When the cells were preincubated with ALS followed by anti-rabbit immunoglobulin, no blocking of the expression of the HL-A antigens could be observed in the microcytotoxicity test (Table 1). When stained with FITC-labelled anti-rabbit immunoglobulin after preincubation with ALS, and reincubated with HL-A antisera followed by rhodamine-labelled anti-human immunoglobulin, the green fluorescence was aggregated into patches and caps in virtually all cells, while the red fluorescence showed a diffuse distribution in the cell membrane, and was superimposed on the green patches and caps. The ALS tested, therefore, neither blocked the expression of the HL-A antigens as measured by microcytotoxicity technique nor induced aggregation of HL-A antigens as determined by immunofluorescence.

To test the specificity of the reactions, highly purified β -2-m (supplied by Dr Bengt G. Johansson, Lund) was added to anti- β -2-m, pooled anti-HL-A and ALS. β -2-m (50 μ g per 0.2 ml antiserum) abolished blocking and aggregation of HL-A and β -2-m determinants by anti- β -2-m as determined by microcytotoxicity test and fluorescence microscopy. The same amount of β -2-m had no influence on the activity of pooled anti-HL-A sera or ALS.

Studies of lymphocyte transformation *in vitro* were carried out by our micromethod¹¹. Results from mixed lymphocyte cultures (MLC) cultured for 5 d in the presence of anti- β -2-m or ALS are shown in Fig. 2. As controls we used partly purified immunoglobulin from rabbits prepared in the same way as the anti- β -2-m antibodies and normal rabbit serum. The results show that anti- β -2-m blocks the MLC reactions (ABm), up to a dilution in normal serum of 1:128. In addition, a very slight but definite mitogenic effect is demonstrated by anti- β -2-m diluted 1:2 (AAm). The ALS shows a much more intense mitogenic effect up to a dilution of 1:32. No definite inhibition of the MLC reactions could be demonstrated by any dilution of the ALS. This is in marked contrast to the results with anti- β -2-m.

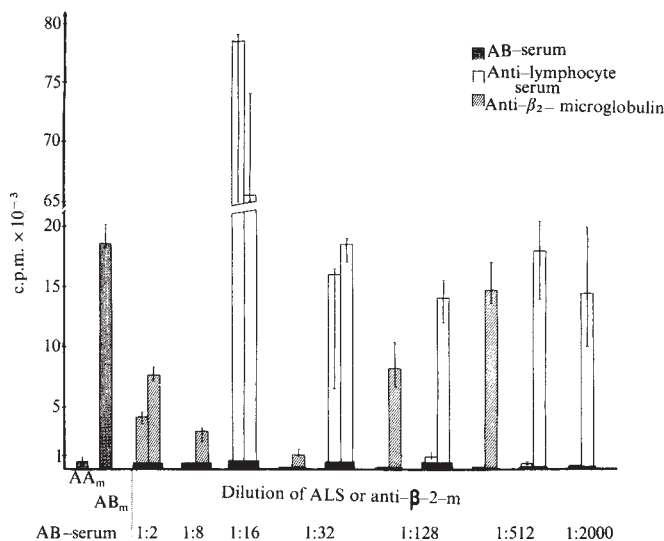


FIG. 2 Effect of different dilutions of anti- β -2-m and anti lymphocyte serum on mixed lymphocyte cultures. 0.15×10^6 responding lymphocytes (from A) were mixed with 0.15×10^6 mitomycin treated stimulating lymphocytes (from either A or B) in the wells of flat bottomed microtitre plates. Each single culture consisted of 0.2 ml Medium 199 supplemented with antibiotics and 20% inactivated normal (AB) or immune serum (diluted in AB-serum). Twenty-four hours before collection, 0.02 ml of $0.05 \mu\text{Ci } 2\text{-}^{14}\text{C}$ -thymidine was added in physiological saline, the cultures collected on glass fibre filters on day 5, and the degree of proliferation evaluated by liquid scintillation counting. The columns indicate the median of the triplicates, and the brackets show the range. The first column in each pair is the autologous combination (AAm) in test serum, the second is the allogeneic combination (ABm) in test serum. The black areas at the bottom of the columns are the autologous control combinations (AAm) in normal serum. Note the mitogenic affect of both anti- β -2-m and ALS, and the inhibitory effect on MLC exhibited by anti- β -2-m only.

Anti- β -2-m was also found to block completely the stimulation induced by PPD of lymphocytes from a BCG-sensitized individual (10 and 100 μg PPD to 0.15×10^6 lymphocytes in 0.2 ml culture medium; collection on day 4). In parallel experiments, anti-HL-A antibodies directed against the lymphocytes had similar inhibitory activity, while an HL-A antiserum not reactive with the lymphocyte donor and normal AB serum did not affect the response. Addition of 50 μg β -2-m per 0.2 ml antiserum abolished the mitogenic and inhibitory activity of anti- β -2-m, but had no effect on the inhibitory effect of anti-HL-A antisera or the mitogenic effect of ALS.

It has previously been shown¹²⁻¹⁴, that antibodies with specificity for HL-A antigens can probably inhibit antigen-induced lymphocyte activation. The results of our present study indicate that anti- β -2-m antibodies also inhibit allogeneic lymphocyte stimulation and lymphocyte transformation induced by PPD. Thus, anti-HL-A and anti- β -2-m antibodies which both induce redistribution and removal of HL-A molecules in the cell membrane, inhibit lymphocyte activation *in vitro*. The xenogenic anti-lymphocyte sera tested showed no activity against HL-A antigens or β -2-m, and did not cause detectable inhibition of lymphocyte activation. A slight inhibitory activity in the anti-lymphocyte sera could be overshadowed by the mitogenic activity demonstrated by these sera. There was, however, definitely no inhibition at serum concentrations being 30 to 60 times lower than the optimal concentration for cap formation at the cell surface. By using anti- β -2-m or anti-HL-A sera^{13,14}, inhibition of lymphocyte activation could be detected at concentrations 30 to 60 times less than those optimal for induction of cap formation. The results with the two anti-lymphocyte sera presented in this study confirm our observations^{13,14} that mixed lymphocyte cultures are not necessarily inhibited by all antibodies induc-

ing aggregation of lymphocyte cell membrane components. Inhibitory activity of other anti-lymphocyte sera on lymphocyte transformation has previously been described¹⁵. This may be due to the presence of anti- β -2-m or other antibodies reacting with the HL-A molecule in these sera.

The efficiency of anti-HL-A and anti- β -2-m sera in inhibiting antigen-induced lymphocyte transformation indicates that both the allo-antigenic subunit and the β -2-m subunit of the HL-A molecule are closely associated with the T lymphocyte receptor. The HL-A molecule may either be part of the T cell receptor, be structurally closely associated with it in the cell membrane, or be functionally associated with lymphocyte activation at a secondary step following antigen binding to the T cell receptor. The convincing homology that has been demonstrated in amino acid sequence between β -2-m and the constant domains of IgG further suggests that β -2-m serves an important effector function in immunological reactions⁴.

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Two classes of inhibitors of peptidyl transferase activity in eukaryotes

THE 12,13-epoxytrichothecenes (Fig. 1) form a group of fungal toxins with a tricyclic trichothecene ring system. (For review, see ref. 1). They have been shown to inhibit protein synthesis in a variety of eukaryotic systems² and in particular they block peptidyl transferase activity as determined by the puromycin-fragment reaction³. More recent studies have shown however, that the trichothecenes have differential effects on protein synthesis in whole cells⁴. One subgroup containing verrucaric acid and T-2 toxin inhibited initiation (I-type), and the other subgroup inhibited elongation/termination (E-type); trichodermin was in the latter group. Here I show that these two classes can also be distinguished in cell-free systems and suggest analogies with the mode of action of the lincosaminide and macrolide inhibitors of protein synthesis in prokaryotes.

It has been observed that the effect of antibiotics on the reaction of puromycin with nascent peptide chains on polyribosomes *in vitro* accurately reflects their effect on trans-

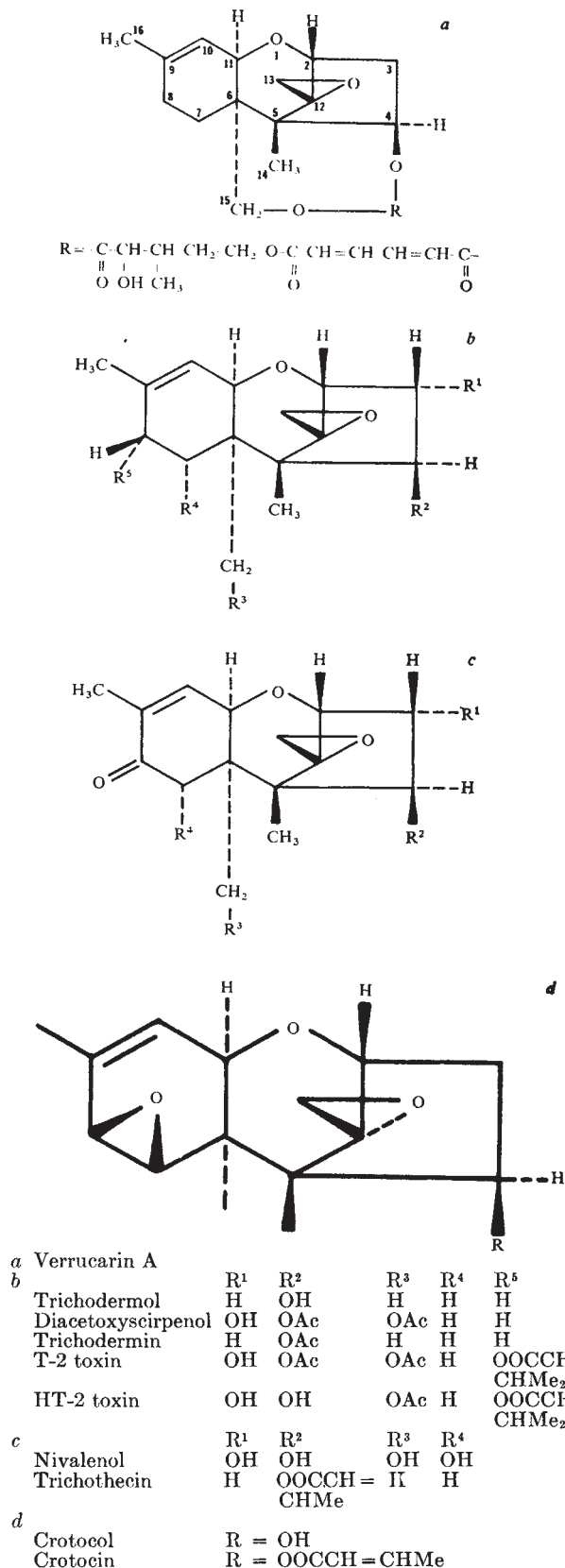


FIG. 1 Structure of some 12, 13-epoxytrichothecenes.

peptidation *in vivo*⁵. The various trichothecene inhibitors were tested using polyribosomes prepared from rat liver, and it was found that the E-type inhibitors, such as trichodermin, inhibit the puromycin reaction, whereas the I-type inhibitors have little or no effect (Table 1). This assay allows the expansion of the E-type subgroup to include crotocin and trichothecin, in addition to trichodermol and trichodermin.

TABLE 1 Inhibition of ³H-peptidyl-puromycin reaction and poly (U)-directed polyphenylalanine incorporation

No drug	³ H-Peptidyl-puromycin		Phenylalanine incorporation	
	μM	% Activity 100% = 2.8 pmol	μM	% Activity 100% = 55 pmol
Trichodermin	35	50	100	87
Trichothecin	35	50	100	70
Trichodermol	80	50	100	98
Crotocin	150	64	100	97
Diacetoxyscirpenol	54	94	80	77
Crotocool	190	91	100	89
Nivalenol	160	106	5.6	50
T-2 Toxin	104	100	4.0	50
Verrucaric A	100	108	1.0	50
HT-2 Toxin	120	96	0.2	50

Effect of various trichothecenes on the reaction of puromycin with rat liver polyribosomes and on poly(U)-directed phenylalanine incorporation. Polyribosomes from rat liver were prepared as described by Baliga *et al.*²⁰. Formation of ³H-peptidyl-puromycin was measured as described previously⁶. Control activity is expressed as pmol of ³H-puromycin incorporated into trichloroacetic acid-precipitable material. ¹⁴C-phenylalanine incorporation using yeast extracts was measured as described previously⁶. The average control activity is expressed as total pmol of ¹⁴C-phenylalanine incorporated into acid-precipitable, alkali stable material.

The I-type inhibitors inhibit poly(U)-directed phenylalanine incorporation on yeast ribosomes⁶. Various trichothecenes were tested in this system and HT-2 toxin behaved like other I-type inhibitors as it inhibited phenylalanine incorporation at low levels and did not affect peptidyl-puromycin formation even at relatively high levels (Table 1). This suggests that HT-2 toxin will inhibit initiation *in vivo*. It can be seen that the E-type inhibitors do not affect phenylalanine incorporation to the same degree as they inhibit the peptidyl-puromycin reaction. Although crotocool and diacetoxyscirpenol were relatively inactive in both *in vitro* assays, diacetoxyscirpenol inhibits initiation *in vivo*⁴. The fact that the I-type inhibitors (verrucarin A, T-2 toxin, and so on) inhibit the puromycin reaction *in vitro* when using CCA-leu (the fragment reaction)⁸ but not the same reaction on polyribosomes is reminiscent of the action of the antibacterial agents lincomycin, certain macrolides such as carbomycin, and the streptogramin A group of antibiotics. These compounds inhibit the puromycin reaction with donors such as CCA-leu in cell-free systems from prokaryotes, but not with nascent peptide chains on polyribosomes; they also seem to inhibit initiation *in vivo*⁸. Further investigation has revealed other similarities between the trichothecenes and the macrolides and lincosaminides.

Surprisingly, although the E-type trichothecenes inhibit elongation *in vivo*, they do not inhibit poly(U)-directed polyphenylalanine synthesis. Similar results have been observed among the macrolides, since erythromycin does not inhibit polyphenylalanine synthesis in a purified *Escherichia coli* system⁷, although it inhibits elongation *in vivo*. On the other hand, spiramycin, an I-type macrolide, does inhibit polyphenylalanine synthesis¹⁰.

The macrolide antibiotic erythromycin does not inhibit the fragment reaction although it inhibits the binding of lincomycin, an antibiotic which blocks the fragment reaction on bacterial ribosomes¹¹. In addition, erythromycin antagonises the inhibition by lincomycin of the puromycin reaction with tRNA^{met} (ref. 12). Lack of inhibition by erythromycin is therefore not the result of an inability to bind to ribosomes under the assay conditions used. I have obtained a similar result with the trichothecenes. Although trichodermin does not inhibit poly(U)-directed phenylalanine incorporation to a large extent, its presence significantly reduces the inhibition produced by I-type inhibitors on yeast ribosomes (Fig. 2a). This suggests that trichodermin is bind-

TABLE 2 Summary of effects of protein synthesis inhibitors on various reactions

	Poly(U)-directed polyphenylalanine synthesis	Puromycin-fragment reaction	Peptidyl-puromycin reaction	<i>In vivo</i>
Eukaryotes				
Verrucarin A	+	+	—	Initiation (ref. 4)
Nivalenol	+	0	—	Initiation (ref. 4)
T-2 toxin	+	0	—	Initiation (ref. 4)
HT-2 toxin	+	0	—	0
Trichodermin	—	+	+	Elongation (ref. 4)
Trichodermol	—	+	+	0
Crotocin	—	0	+	0
Trichothecin	—	+	+	0
Prokaryotes				
Lincomycin	+	+	—	Initiation (ref. 8)
Spiramycin	+	+	—	Initiation (ref. 8)
Erythromycin	—	—	—	Elongation (ref. 9)

+, Inhibition; —, No inhibition; 0, not done.

ing to the ribosome and preventing the binding of the other inhibitors. This may indicate partially overlapping binding sites on the ribosome, although other possibilities cannot be excluded, such as the binding at one site producing a conformational change at another site. But the similarity in structure between trichodermin and HT-2 toxin, for example, strongly suggests a common site of binding.

The converse experiment was carried out using varying levels of verrucarin A and trichodermin in a puromycin reaction on rat liver polyribosomes (Fig. 2b). The highest concentration of verrucarin A (40 μM) did not antagonise inhibition by trichodermin, whereas the same concentration of verrucarin A inhibits poly(N)-directed polyphenylalanine incorporation by 90%. This could be taken as evidence for binding sites which are not mutually exclusive, but this would conflict with the results of the previous experiment which suggested a common binding site. A more likely possibility is that peptidyl-tRNA prevents the binding of verrucarin A to ribosomes as previously suggested by Cundliffe *et al.*⁴. On stripped ribosomes both E- and I-type inhibitors might bind to mutually overlapping sites. This explains why the I-type inhibitors prevent either initiation or steps shortly after initiation of protein synthesis and not elongation, thereby permitting run-off of polysomes. A similar explanation has been used to explain why the lincosaminides do not inhibit elongation of peptide chains on bacterial polyribosomes¹³. Since verrucarin A does not inhibit the puromycin reaction on salt-washed 80S yeast ribosomes, while trichodermin

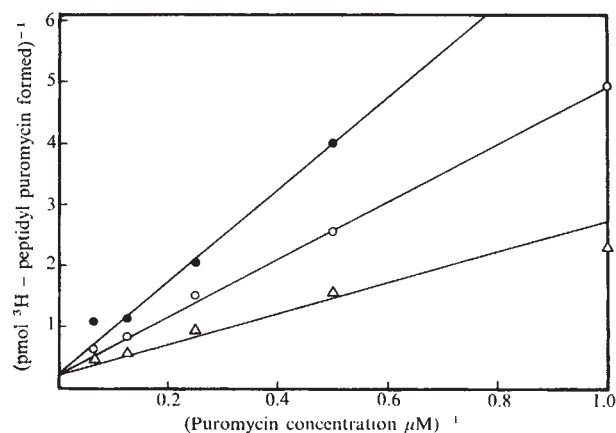


Fig. 3 Competitive inhibition of ^3H -peptidyl-puromycin synthesis by trichodermin. Assays were performed as described previously but incubations were carried out for 1 min to provide initial rates. ●, Trichodermin (68 μM); ○, trichodermin (17 μM); Δ, no trichodermin.

does (unpublished observations), one can probably rule out the involvement of any factor(s) present on unwashed ribosomes which may prevent the binding of verrucarin A.

Further evidence for overlapping sites comes from the fact that a trichodermin resistant strain of *S. cerevisiae*⁶ is also resistant to T-2 toxin (100 $\mu\text{g ml}^{-1}$) and anisomycin (20 $\mu\text{g ml}^{-1}$). Similarly, bacterial mutants resistant to erythromycin are known to be resistant to lincomycin, spiramycin, and the streptogramin B antibiotics¹⁴.

The nature of the inhibition of the puromycin reaction on rat liver polyribosomes by trichothecenes is of some interest. When plotted on a double reciprocal plot, trichodermin seems to be competitive inhibitor of the puromycin reaction. The K_m for puromycin was 13×10^{-6} M which compares to 8×10^{-6} M previously reported by Pestka¹⁵. Carbomycin, tylosin, spiramycin, and lincomycin, all I-type macrolides, inhibit binding of CACCA-Phe to prokaryotic ribosomes¹⁶, a model reaction for the interaction of the acceptor aminocyl-tRNA to the peptidyl transferase centre. Trichodermin, trichodermol, and anisomycin inhibit binding of both CACCA-LeuAc and UACCA-Leu to ribosomes, presumably by preventing access of these aminoacyl-tRNA fragments to the donor and acceptor sites of the peptidyl transferase centre³. But inhibition of this binding was found to be less than inhibition of the puromycin fragment reaction, which may be due to different experimental conditions in the two assays. The competitive inhibition (elevating the K_m but not changing the V_{max}) which I observe, indicates weaker binding of puromycin and is consistent with blockade of the binding at the acceptor site in the peptidyl transferase centre by tri-

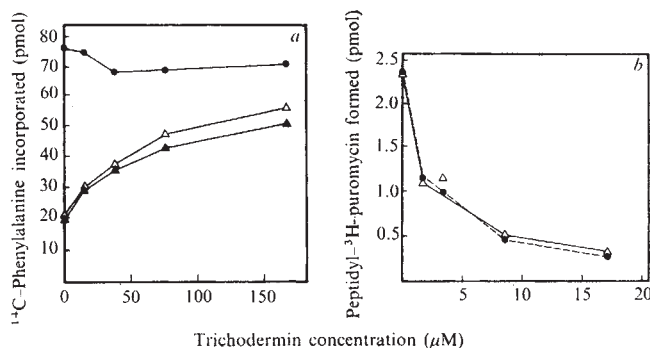


Fig. 2 a, Antagonism by trichodermin of the inhibition of poly U-directed phenylalanine incorporation by I-type inhibitors. ^{14}C -phenylalanine incorporation was measured as described previously⁶. Activity is expressed as total pmol of ^{14}C -phenylalanine incorporated into alkali-stable, acid-precipitable material in 30 min. Similar results were obtained using nivalenol (8 μM) and T-2 toxin (2 μM). ▲, Verrucarin A (2.0 μM); Δ, HT-2 toxin (2.4 μM); ●, control. b, Lack of antagonism by verrucarin A of the inhibition of ^3H -peptidyl-puromycin formation of trichodermin. ^3H -Peptidyl-puromycin was measured as described in Table 1. Δ, Verrucarin A (40 μM). ●, control.

chodermin under conditions more closely resembling protein synthesis (for example, absence of ethanol).

I suggest that the I-type trichothecenes, macrolides, and lincosaminides are similar in their mode of action of protein synthesis inhibition. The basis for the proposed analogy is summarised in Table 2. It fails at one important point for the E-type inhibitors in that the E-type macrolides, such as erythromycin, and the E-type trichothecenes differ in their effects on the peptidyl-puromycin and the puromycin-fragment reactions.

The macrolides which inhibit initiation *in vivo* have been subdivided into two classes based on the size of oligophenylalanine produced in their presence in a poly(U)-directed system⁷; it would be interesting to see if the I-type inhibitors of the trichothecenes can be classified in the same manner. Diacetoxyscirpenol may be a member of a second class of I-type inhibitors because of its anomalous effect on poly(U)-directed polyphenylalanine synthesis.

It has been postulated that the presence of substituents on carbon 15 of the trichothecene ring system may be important in determining whether the toxin will affect elongation or initiation⁴. The assignments of crotoxin and trichothecin to the class of E-type inhibitors and HT-2 toxin to the class of I-type inhibitors based on *in vitro* experiments further substantiates this hypothesis. HT-2 and T-2 toxin are substituted at C-15; both crotoxin and trichothecin, like other E-type inhibitors, lack a substituent at this position.

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Effect of insulin on (Na⁺, K⁺)-activated adenosine triphosphatase activity in rat muscle sarcolemma

THE existence of (Na⁺, K⁺)-activated adenosine triphosphatase (ATPase)^{1,2} supports a molecular explanation for the active transport of sodium and potassium ions through cell membranes³.

Other than results from insulin stimulation of membrane-bound ATPases from human lymphocytes⁴, no studies have, so far, given any proof of connecting insulin effects on electrolyte transport through cell membranes with the effect on the ATPases. Our observations show an insulin stimulation of sarcolemma-bound ATPase when muscle tissue is incubated with insulin before measuring the enzyme activity on isolated membrane preparations.

Wistar albino rats weighing 170-250 g were fasted for 20-22 h. The rats were killed with a sharp blow to the head and exsanguinated or decapitated when in ether narcosis. In other experiments the animals were anaesthetised with Nembutal sodium (30 mg kg⁻¹, at 30 min intervals) for 1½ h, then bled by cardiac puncture for sugar analysis⁵ and decapitated. The leg muscles were excised, transferred to ice-cold 0.9% NaCl, blotted on filter paper and weighed. In the first series, the sarcolemma was isolated according to the method of Severson *et al.*⁶, and the membrane preparations incubated with insulin before enzyme assay. In the other experiments, muscle pieces were incubated with insulin (100 mU ml⁻¹) for 15 min in Tyrode's solution⁷ before membrane isolation⁶. The membrane pellets from all experiments were resuspended in 0.01 M Tris HCl buffer (pH 8.0) approximately five times the original tissue volume (that is, tissue weight) and subjected to two strokes in a Potter-Elvehjem glass homogeniser with a motor-driven Teflon pestle (5,200

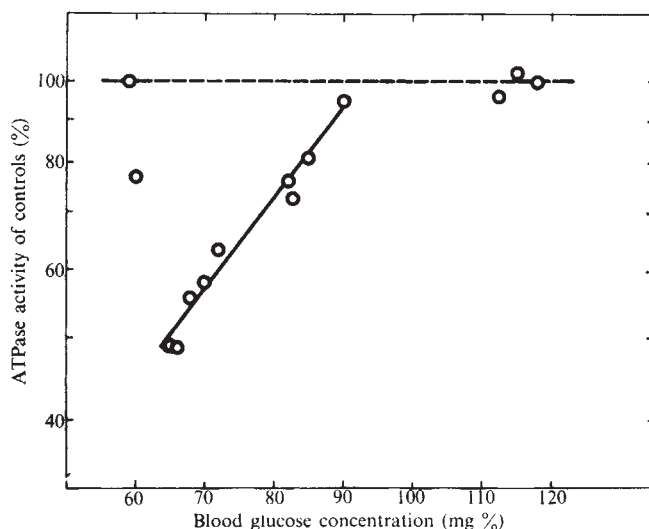


FIG. 1 Semilogarithmic plot of ATPase activity. ●, Variations in specific activities of the controls (%) correlated with blood sugar concentration (mg% whole blood). --- The specific activities after insulin treatment were taken as 100%; in these experiments only Nembutal-treated rats were used.

TABLE 1 Effect of insulin on isolated muscle sarcolemma ATPase and 5'-nucleotidase activities

Enzyme	No. of experiments	Specific activity $\pm$ s.e.m. ($\mu\text{mol P}_i$ per mg protein per min) $\times 10^2$	
		Without insulin	With insulin
(Na ⁺ , K ⁺)-ATPase	15	22.2 $\pm$ 1.8	23.3 $\pm$ 1.9
5'-nucleotidase	6	2.12 $\pm$ 0.15	1.91 $\pm$ 0.14

ATPase and 5'-nucleotidase activities at 30° C as liberation of inorganic phosphate from ATP and AMP, respectively. Isolated muscle sarcolemma was preincubated with and without 100 mU ml⁻¹ pig insulin at 37° C for 15 min. The membrane preparations were then homogenised in 0.01 M Tris HCl buffer, pH 8.0. For the ATPase assay 500 μl of the membranes was mixed with 650 μl Tris HCl buffer, final pH 7.6, containing NaCl, KCl, and MgSO₄, giving a final concentration of 30, 5, and 0.5 mmol l⁻¹, respectively, after substrate addition. The reaction was started by adding 500 μl 5 mmol l⁻¹ ATP and stopped after 2 min by addition of 0.5 ml ice-cold 15% trichloroacetic acid. For the assay of 5'-nucleotidase, 400 μl membrane homogenate and 600 μl Tris HCl buffer, pH 8.0, with MgCl₂ and AMP to a final concentration of 80 mmol l⁻¹, 0.2 mmol l⁻¹, and 2 mmol l⁻¹, respectively. The reaction was stopped after 10 min. Reaction vessels were centrifuged and the supernatant analysed for phosphate⁸. The precipitate was solubilised in 5 ml 3% deoxycholic acid sodium salt, warmed at 80° C for 5 min and protein content estimated⁹ using a bovine serum albumin as standard. Every enzyme sample was run in triplicate.

r.p.m.). The homogenates were used to assay (Na⁺, K⁺)-activated ATPase and 5'-nucleotidase, as liberation of inorganic phosphate⁸ per mg protein⁹ per min.

In the third series, non-fasted rats were rendered diabetic by intravenous injection of 55 mg alloxan in a 5% aqueous solution per kg body weight. The severity of diabetes was assessed by measuring the blood glucose⁵ 48–72 h after treatment. Blood sugar was sampled at the time the animals were killed. Those having a plasma glucose concentration exceeding 450 mg % were used in the experiments.

Attempts at direct stimulation of the membrane ATPases with insulin did not succeed (Table 1), contrary to the results of Hadden *et al.*⁴ Varying the incubation time, temperature, ionic environment, insulin concentration, and preincubation with adrenaline did not alter this. The sample to be examined was compared with a control from the same animal. When muscle cells were preincubated with insulin, ATPase activity was stimulated in the muscle sarcolemma, but the specific activity of the 5'-nucleotidase was unaffected (Table 2).

Although all rats in each series received the same treatment, we obtained great variations in percentage stimulation of ATPase activities. The hormone-stimulated values were quite consistent, but the controls showed great deviations (Table 2). This might indicate that ATPase activity is governed by circulating insulin concentration. If the animals are stressed during handling, there is an increase in blood sugar, followed by an insulin response¹⁰ which, in turn, stimulates ATPase activity. In an attempt to relax the animals, one series of experiments was carried out with the animals anaesthetised with Nembutal. Blood glucose concentration

was used as an indication of the circulating insulin level¹¹.

Figure 1 shows the variations in ATPase activity of the controls, expressed as an exponential function of blood sugar concentration. Insulin stimulation fails when the specific activities approach the maximum level (Fig. 1). The exceptions at the lower sugar concentrations can be explained if one assumes that these low sugar levels result from high circulating insulin concentrations and, consequently, stimulate the ATPase activity, abolishing further stimulation *in vitro*.

The results obtained with muscle tissue from alloxan diabetic rats (Table 2) after *in vitro* insulin treatment show great variation in the stimulation of the ATPase activity, ranging from more than 100% to 0%. When hormone stimulation fails, the specific activity of the control samples is found at the same upper level as those which respond to insulin stimulation. Alloxan in a diabetogenic dose shows a triphasic response, with high insulin secretion in the last phase¹². Time dependence for reaching nullity of circulating insulin is subjected to great individual variations¹³. Disregarding the irregular data, these results support our hypothesis.

Different types of ATPase activities are recognised within cell membranes. Whether they represent partial activities of the same enzyme system or entirely different enzymes, is still a matter of dispute. The (Na⁺, K⁺)-activated ATPase has an active site for K⁺ outside and an active site for Na⁺ inside the cell membrane, and the two ions compete with each other on both sites^{3,14}.

Omitting K⁺ in the enzyme assay resulted in a residual activity of about 8% of controls¹⁵. This was taken as the criterion for the assumption that the membrane-bound ATPase in question is the (Na⁺, K⁺)-activated ATPase. Dialysis of sarcolemma preparations against 0.01 M Tris HCl buffer, pH 8.0, resulted in a further loss of activities when K⁺ was omitted (unpublished data). Omitting both monovalent cations from the enzyme assay resulted in an ATPase activity of 60–70% of that obtained under complete assay conditions, consistent with previous findings^{6,16}. We have chosen not to subtract any of these residual or partial activities, because we were able to detect an insulin effect on the ATPase in conditions we consider more 'physiological'.

Our results were obtained with rather high insulin concentrations. The data from a few experiments with physiological hormone concentrations did not show any significant enhancement of the ATPase activity (unpublished data). We interpreted this as a built-in shortage of the *in vitro* system; instead of vascular transport of insulin to the muscle cells, it is distributed within the muscle mass by means of a concentration gradient. When muscle pieces are mechanically divided longitudinally, a leakage of insulin-degrading enzymes from broken cells might occur, which would destroy fractions of the hormone, the degradation being more pronounced, the lower the hormone concentration^{17,18}. In addition, insulin might adsorb to glassware. Different species of insulin might also influence the response.

TABLE 2 Effect of insulin on sarcolemma ATPase and 5'-nucleotidase activities after *in vitro* incubation of muscle cells with insulin

Enzyme	Treatment of animals	No. of experiments	Specific activity $\pm$ s.e.m. ($\mu\text{mol P}_i$ per mg protein per min) $\times 10^2$	
			Without insulin	With insulin
(Na ⁺ , K ⁺)-ATPase	Ether anaesthesia	15	28.7 $\pm$ 2.9	33.5 $\pm$ 3.2
(Na ⁺ , K ⁺)-ATPase	Nembutal	10	21.2 $\pm$ 1.5	29.2 $\pm$ 0.6
(Na ⁺ , K ⁺)-ATPase	Alloxan diabetic	5	21.0 $\pm$ 3.8	28.0 $\pm$ 0.6
5'-nucleotidase	Nembutal	11	2.03 $\pm$ 0.13	1.99 $\pm$ 0.16

ATPase and 5'-nucleotidase activities assayed at 30° C as liberation of inorganic phosphate from ATP. Except for the alloxan diabetic rats, the animals were fasted for 20–22 h. Small muscle pieces were incubated in cylindrical vessels (1.6 $\times$ 10 cm) containing 2.5 ml Tyrode solution (8.0 g NaCl, 0.2 g KCl, 1.0 g CaCl₂, 0.05 g MgCl₂, 0.05 g NaH₂PO₄, 1.0 g NaHCO₃, and 2.0 g glucose diluted to 1 l), flushed with O₂:CO₂ = 95%:5%. Tissue mass per tube was 1.5 g. After temperature equilibrium, 10 μl insulin (1.2 mg ml⁻¹) was added to the incubation medium. Samples and controls were incubated at 37° C for 15 min. The controls and samples were treated equally and isolated simultaneously. The enzyme activities were measured as in Table 1.

It seems reasonable to conclude that insulin stimulates ATPase activity under physiological conditions, as shown indirectly by the variation of the controls as a function of blood sugar concentrations (Fig. 1), and from the fact that the highest control values are not further increased by incubation with high insulin concentrations *in vitro*.

Although this technique is adequate to demonstrate an insulin effect on membrane-bound ATPase activity, a better system must be developed in order to gain an insight into the mechanism of action.

The effect of insulin on stimulation of Na⁺ and K⁺ transport through muscle cell membranes is long established^{19,20} but no attempts to link this effect to an insulin stimulation of the (Na⁺, K⁺)-activated ATPase have succeeded^{2,21}. There might be different reasons for this: the enzyme activity has been assayed with crude cell homogenates in which the insulin effect is masked by gross action of unspecific phosphatases (unpublished results); isolated membrane preparations were exposed to the hormone before the enzyme was assayed without any effects, in agreement with our results, or controls might have been stimulated by insulin release after stress when the animals were killed.

Without any transport data, our experiments led to three conclusions. First, the (Na⁺, K⁺)-activated ATPase from isolated muscle membranes is not stimulated directly by insulin. Second, incubation of the muscle tissue with insulin before isolation of the membranes resulted in an increased ATPase activity and, third, there seems to be a correlation between blood sugar concentration and the tendency to stimulate ATPase activity *in vitro* by insulin.

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In vitro suppression of cell mediated autoimmunity in NZB mice

THE spontaneous autoimmune disease of NZB mice is characterised by the development of a variety of autoantibodies, particularly those reacting with autologous erythrocytes as demonstrated by a positive direct Coombs test. The onset of autoantibody production coincides with thymic hyperplasia and is followed by splenomegaly and glomerulonephritis¹. All the tissues contain murine leukaemia virus particles (type C) visible on electron microscopy² and Gross antigen is detectable immunologically³. This virus is vertically transmitted, yet antibodies have been shown to develop to Gross antigen at the time the direct Coombs test becomes positive⁴. The role of this virus, if any, in the aetiology of the autoimmune features is still unclear but it has been suggested that this progressive loss of tolerance to Gross antigen is of pathogenetic importance^{4,5}.

Old surviving mice have a high frequency of lymphomata^{6,7}. Many of these are transferable to other mouse strains, but autoimmune phenomena are not associated with these^{6,8}. It has not been possible to reliably transmit the disease to other strains with a cell-free filtrate nor indeed with whole cells⁹⁻¹¹ unless sufficient immunosuppression was also used to convert the recipient into an effective chimera¹². A depression of cell-mediated immunity to certain extrinsic antigens has been demonstrated in these mice before the manifestation of the autoimmune phenomena^{13,14}, yet the proportion of θ -positive cells in the spleen was shown to remain essentially undiminished^{15,16}, although one report does describe a progressive decrease with age¹⁷. Recently, we have provided evidence which suggests loss of the homeostatic control of autoreactivity with respect to cell mediated immunity: lymphocytes from diseased adult NZB mice were cytotoxic *in vitro* to syngeneic or autochthonous foetal fibroblasts. Cells from young NZB mice did not show this reactivity^{18,19}. We here provide evidence that this cytotoxicity depends on the presence of θ -positive, presumably thymus-derived, cells. Furthermore, we were able to show inhibition of the cytotoxic effect of lymphocytes from the Coombs-positive, diseased NZB mice (NZB⁺) by non- θ -bearing lymphocytes from young, Coombs negative NZB mice (NZB⁻), and from young mice of other strains.

A modification of the microcytotoxicity assay of Takasugi and Klein²⁰ was used as previously described¹⁸. Test fibroblasts were cultured in Falcon microtest plates at a concentration of 100 cells per well and 24 h later, splenic lymphocytes purified on a Ficoll/Isopaque gradient²¹ were added.

In these conditions, lymphocytes from NZB⁺ mice were cytotoxic to syngeneic or autochthonous foetal NZB fibro-

TABLE 1 Effect of treatment of lymphocytes with anti- θ antiserum and fresh complement before incubation of these cells with fibroblast monolayers.

Source of lymphocytes*	Pretreatment	Source of fibroblasts	No. of Fibroblasts Remaining ($\pm$ s.e.m.)
NZB+	nil	NZB foetal	43 $\pm$ 3†
NZB-	nil	NZB foetal	115 $\pm$ 8†
NZB+	Anti-theta serum and C'	NZB foetal	132 $\pm$ 19
NZB	Anti-theta serum and C'	NZB foetal	130 $\pm$ 8
CBA	nil	NZB foetal	93 $\pm$ 7
NZB+	nil	CBA foetal	74 $\pm$ 7
NZB-	nil	CBA foetal	87 $\pm$ 6
CBA	nil	CBA foetal	97 $\pm$ 9

* NZB+, Autoimmune Coombs positive NZB mouse; NZB-, normal Coombs negative NZB mouse (see text); C', guinea pig complement absorbed with agarose (see text)

† Significantly different from control values (P < 0.001)

‡ Control values

TABLE 2 Effect on autoreactivity of NZB+ cells by preincubation with dilutions of anti- θ antiserum with and without complement.

Dilution of anti- θ antiserum	Complement	% fibroblasts remaining* ($\pm$ s.e.m.)†	
—	—	100	(± 7)*
1:1	+	145	(± 4)
1:2	+	96	22
1:5	+	106	9
1:10	+	43	5
1:1	—	31	3†
1:2	—	18	2†
1:5	—	19	2†
1:10	—	16	2†
—	+	10	3
—	—	30	5§
—	—	6	1

* Expressed as a % of control value obtained without addition of lymphocytes.

† Converted to a percentage.

‡ Significantly different from results where complement was included.

§ Preincubation with medium alone.

|| No incubation.

blasts as is seen in Table 1. As our tissue culture system used heat-inactivated foetal calf serum in the culture medium, this phenomenon was most likely the result of cell mediated autoreactivity. In our previous experiments, no significant toxicity was demonstrable against fibroblasts from neonatal or adult BALB/c and CBA mice¹⁸. The ratios of lymphocytes to fibroblasts were varied from 1:10 to 1:2,000. The dose response curve obtained, showed that near maximal killing was achieved with a cell number ratio of 1:200 (ref. 18). In keeping with the methodology developed by Hellstrom²², experiments were carried out using a ratio of one fibroblast to 2,000 lymphocytes.

The cellular origin of the NZB-killer activity was assessed by testing for the possible involvement of θ -bearing thymus derived cells. Lymphocytes from NZB+ mice were treated with anti- θ serum and complement as described elsewhere²³. The potency of this antiserum was verified by its capacity to interfere with the *in vitro* immune response to a T-cell dependent antigen (sheep erythrocytes) but not to a T-cell independent antigen (polymerised flagellin from *Salmonella adelaide* strain 1338). The source of complement was guinea pig serum previously absorbed with agarose for 30 min at 4° C, according to the method of Cohen and Schlesinger²⁴. Such serum was free of non-specific cytotoxicity and had no effect on the potency of 'killer' cells from NZB+ mice. Results from a representative experiment shown in Table 1 indicate that the cells involved in the 'self' killing mechanisms bear the θ antigen. The result of diluting the anti- θ antiserum can be seen in Table 2. It is only effective in the presence of complement. Cohen²⁵ has previously described

the development *in vitro* of cell mediated autoimmunity. The responsible cells here too seem to be of thymic origin.

We have previously reported that the *in vitro* killing of NZB fibroblasts by NZB+ lymphocytes may not be inhibited by serum from young NZB- mice²⁶. It seems that the mechanisms which must prevent *in vivo* autoreactivity in the clinically normal, NZB- mouse are mediated by either serum factors too weak to be detected *in vitro* or by direct cellular control. To try and re-establish control over the autoimmune response *in vitro*, lymphocytes from NZB+ mice were mixed with cells from 4-week-old mice before being added to the fibroblasts. Table 3 demonstrates that substitution of even 10% of cells from NZB- mice significantly suppressed the autoreactivity of lymphocytes from diseased NZB mice. This suppression effect was also demonstrable with cells from both BALB/c and CBA/HI strains. In each case, the effect of NZB- cells alone was taken as the control but each experiment must be considered individually as different lymphocytes have a variable stimulating effect on the growth of the fibroblasts present. It is therefore difficult to compare directly the relative efficacies of different cell types in their capacity as suppressor cells in this system. One may argue that inhibition of fibroblast killing by cells from clinically normal mice could have been due to a dilution effect on the killer cells. It is also possible that NZB- cells added to

TABLE 4 The effect of pretreating young NZB lymphocytes with anti- θ antiserum and complement on the inhibition of the autoimmune response.

Proportion of young NZB cells in mixture*	Pretreatment	Proportion of old cells	No. of fibroblasts remaining ($\pm$ s.e.m.)
—	—	100%	43 $\pm$ 3†
100%	—	0%	115 $\pm$ 8†
10%	—	90%	93 $\pm$ 7
10%	+	90%	111 $\pm$ 11
20%	+	80%	128 $\pm$ 10
33%	+	66%	132 $\pm$ 9
50%	+	50%	111 $\pm$ 11
20%§	—	80%	49 $\pm$ 4†

* Total number of cells (200,000) taken as 100%

† Significantly different from control value $P < 0.001$

‡ Control value

§ Killed by heating at 56° for 30 min

the autoreactive lymphocytes have acted as alternative targets and may thereby have competed with target fibroblasts for killer cells.

These suggestions seem untenable for the following reasons. First, the reduction in total number of self reactive cells from 200×10^3 to 180×10^3 or even 20×10^3 had little effect on reducing fibroblast killing¹⁸ indicating that there was a gross excess of 'killer' cells when 200×10^3 lymphoid cells from NZB+ mice were added to the 100 fibroblasts in a single well. Second, heat killed lymphocytes no longer inhibited the autoreactivity of NZB+ cells (Table 4).

If the NZB- cells responsible for the suppression phenomenon were theta-bearing thymus-derived cells, suppression of autoreactivity should no longer be demonstrable after treatment of these cells with anti-theta serum and complement. As seen in Table 4, however, preincubation of young NZB cells with these reagents failed to abolish the *in vitro* suppressive effect. We tentatively suggest, therefore, that the observed suppression of self reactivity is mediated by θ negative bone-marrow derived B cells rather than by thymus-derived T cells. This is in accord with the similar finding of Phillips and Wegmann²⁷ on tetraparental mice. The possibility is not ruled out, however, that T cells with a low density of the θ surface marker may have escaped the cytotoxic action of anti- θ serum and complement.

Suppression of cell-mediated immunity by humoral factors

TABLE 3 The effect of the addition of lymphocytes from young animals on the cytotoxic ability of cells from diseased NZB mice on their isogenic fibroblasts.

Source of young	No. Added	No. of NZB+ cells	No. of fibroblasts remaining ($\pm$ s.e.m.)
—	—	200,000	53 $\pm$ 6*
NZB—	20,000	180,000	143 $\pm$ 13
NZB—	40,000	160,000	154 $\pm$ 21
NZB—	200,000	—	128 $\pm$ 20†
BALB/c	20,000	180,000	68 $\pm$ 6*
BALB/c	40,000	160,000	109 $\pm$ 7
BALB/c	200,000	—	99 $\pm$ 9*
CBA/HI	20,000	180,000	123 $\pm$ 19
CBA/HI	40,000	160,000	196 $\pm$ 31
CBA/HI	200,000	—	156 $\pm$ 20†

* Significantly different from control values ($P < 0.01$).

† Control values

is now well known in similar experimental situations to the above. We have suggested earlier on experimental grounds that blocking of immunocompetent cells by antigen-antibody complexes could account for mechanisms of self tolerance²⁸. As discussed elsewhere, immunocompetent cells could conceivably become inactivated *in vivo* by antigen-antibody complexes in solution or when attached to the surface of macrophages²⁹. Depending on the configuration of a complex (as determined by the relative amounts of complexed antigen and antibody) a matrix of closely spaced free antigenic determinants of identical specificity would provide the optimal conditions for lymphocyte inactivation. In the case of tissue fixed complexes *in vivo*, blocking activity in serum may be minimal or totally absent and this could account for our failure to demonstrate such activity in the serum of NZB- mice²⁴. Furthermore, inhibition *in vitro* of self reactivity by the addition of small numbers of lymphocytes from healthy animals could be due to the release of B cell derived 'auto-antibody' becoming complexed with a fibroblast derived 'self' antigen. These complexes would readily interact directly with immunocompetent effector cells by blocking their reactivity. A key question pertains to the mechanisms responsible for the breakdown of self tolerance in NZB mice as well as to the nature of the relevant antigen in this case. Although our work to date fails to provide information on these points, it opens a new experimental approach to the investigation of self tolerance and its homeostatic control.

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Intracellular J chain in mouse plasmacytomas secreting IgA, IgM and IgG

THE final step in assembly of IgA and IgM is the polymerisation of intracellular monomeric (H_2L_2) subunits. This event takes place just before, or at the time of secretion¹⁻⁷, and involves the incorporation of J chain^{8,9} into IgM¹⁰ and polymeric, but not monomeric IgA¹⁰⁻¹¹. An unanswered question is whether J chain travels through the membranous elements of the cell, as do the other immunoglobulin subunits¹². As a prelude to answering this, we have demonstrated intracellular J chain in polymer-secreting mouse myeloma cells. In addition, the postulation of an intracelonal switch from IgM to IgG in B-lymphocytes¹³ prompted us to look for, and find, J chain in cells which secrete IgG.

To detect intracellular J chain, mouse myeloma cells were grown *in vitro* in the presence of radioactive leucine¹. The cells were lysed either with 1% (w/v) Nonidet P40 or 1% (w/v) sodium deoxycholate in the presence or absence of 0.1 M iodoacetamide. Following centrifugation (30,000g, 40 min), a rabbit antiserum reactive with free, but not bound, J chain (R.M.E.P., unpublished) was added to the cell lysate, and complexes of radioactive J chain and the rabbit antibody were precipitated with goat anti-rabbit IgG as previously described¹. The antibody-antigen precipitates were washed, reduced and alkylated and electrophoresed in alkaline urea polyacrylamide gels^{10,14}, and the amount of radioactivity in J chain was measured as the total found in the J chain peak.

Initial experiments were done with a tissue culture-adapted cell line of MOPC 315 which produces IgA and which was found to secrete 70% of the myeloma protein in dimer form; the remaining myeloma protein consisted of monomer and higher polymers in approximately equal amounts. With this cell line, similar yields of radioactivity in intracellular J chain were obtained whichever of the methods outlined above was used for lysing the cells.

The results of a typical experiment are presented in Fig. 1. When the total radioactive cell lysate was electrophoresed in an alkaline urea gel (Fig. 1a) there was no discrete peak of J chain. The presence of intracellular J chain could only be demonstrated in the precipitate formed with the specific anti-J chain reagent (Fig. 1b). Electrophoresis of unlabelled cell lysates of MOPC 315 myeloma cells followed by staining of the polyacrylamide gel, however, has been reported to reveal a prominent band in the J chain position¹⁵. In similar experiments using 6% (w/v) alkaline urea polyacrylamide gels we were unable to demonstrate J chain in MOPC 315 myeloma cell lysates, and we would therefore conclude that the reported prominent J chain band¹⁵ is contaminated by other proteins.

To be certain that the peak of radioactivity in the J chain position (Fig. 1b) is authentic, and not the result of another cellular protein with similar electrophoretic properties, the radioactive cell lysate was reacted with a sample of anti-J chain antiserum which had been previously absorbed with a highly purified preparation of mouse J chain¹⁶. The re-

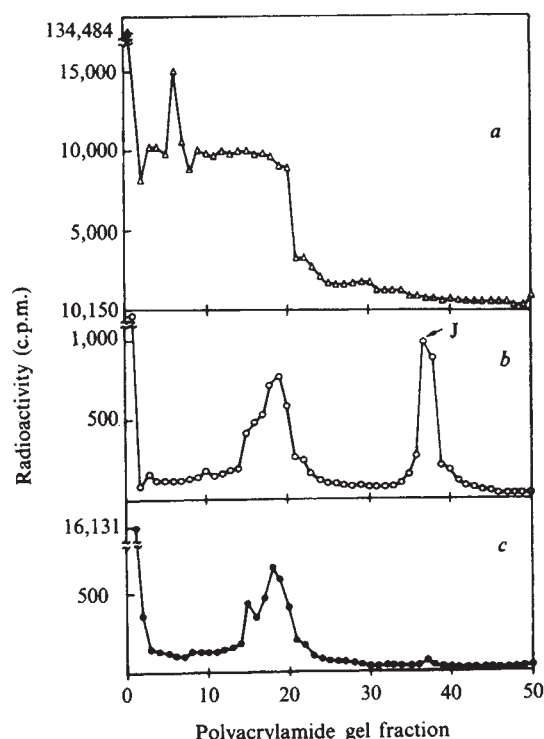


Fig. 1 Identification of J chain in cell lysates of tissue culture-adapted MOPC 315 mouse myeloma cells. Analysis by polyacrylamide gel electrophoresis. Myeloma cell suspensions at 2×10^7 cells ml^{-1} were incubated for 2 h in Eagle's medium containing 0.2 mCi of L-4,5- ^3H -leucine (51 Ci mmole^{-1}) ml^{-1} . The cells were collected by centrifugation, washed once with tissue culture medium and lysed in 0.075 M NaCl-0.075 M Tris-HCl, pH 8.0 containing 1% (w/v) sodium deoxycholate, 0.1 M iodoacetamide and DNase ($50 \mu\text{g ml}^{-1}$). After standing at 37°C for 10 min, the cell lysates were centrifuged at $30,000g$ for 40 min and the supernatants were reserved for analysis on alkaline-urea 6% (w/v) polyacrylamide gels^{10,14}. Before electrophoresis of the total unfractionated cell lysate, the material was dialysed against 8 mM Tris-25 mM glycine, pH 8.7. Radioactive J chain in the cell lysate was isolated by precipitation of soluble complexes formed between rabbit anti-mouse J chain and intracellular J chain with goat anti-rabbit immunoglobulin. As a control precipitation, a sample of the rabbit anti-mouse J chain was first absorbed with an excess of purified J chains¹⁰ and then added to the radioactive cell lysate for precipitation with goat anti-rabbit immunoglobulin. Antibody-antigen precipitates were washed four times with phosphate-buffered saline containing 1% (w/v) Nonidet P-40, once with 8 mM Tris-25 mM glycine, pH 8.7, and then dissolved in 9 M urea-10 mM dithiothreitol by heating for 2 h at 56°C . Samples were then applied to alkaline-urea (6% (w/v)) polyacrylamide gels. The gels were sliced, dissolved and counted¹⁰, and fractions are numbered from the negative (top) to the positive electrode. *a*, Total cell lysate; *b*, radioactive protein in the cell lysate precipitated by the rabbit anti-(mouse J chain) antiserum; *c*, radioactive protein in the cell lysate precipitated with the rabbit anti-mouse J chain antiserum which had been previously absorbed with purified mouse J chain.

sulting antibody-antigen precipitate between rabbit IgG and goat anti-rabbit IgG no longer contained radioactive J chain (Fig. 1c), thereby unequivocally confirming the original identification (Fig. 1b). The problem of non-specific contamination of the antibody-antigen precipitate is overcome by taking advantage of the unique electrophoretic mobility of J chain. The amount of radioactivity coprecipitated by absorbed and non-absorbed anti-mouse J chain is essentially the same, and only when the electrophoretic analysis is done can J chain be identified and quantitated.

The absence of a covalent linkage between intracellular J chain and the intracellular monomer precursor of secreted polymer^{7,10} was confirmed with the tissue culture form of

MOPC 315 myeloma cells, taking advantage of the specificity of the myeloma protein for 2,4-dinitrophenol. Cells ($2 \times 10^7 \text{ ml}^{-1}$) were labelled for 1 h with ^3H -leucine (59 Ci mmol^{-1} , 0.2 mCi ml^{-1}), and the resulting cell lysate (1% w/v Nonidet P-40, 0.1 M iodoacetamide) was filtered through a column of ϵ -N-2,4 dinitrophenyl-lysyl-sepharose¹⁷. The eluate was examined for free J chain by precipitation with the specific antiserum. The intracellular monomeric IgA was eluted from the column with 0.1 M DNP-glycine, reduced (10 mM dithiothreitol) and then electrophoresed in alkaline urea polyacrylamide gels to determine the presence or absence of associated J chains. Free J chain was found in the filtrate from the column and accounted for $1.5 \times 10^{-2}\%$ of the total radioactivity precipitated by 10% (w/v) trichloroacetic acid; there was no detectable J chain associated with intracellular myeloma protein, although 10^5 c.p.m. were applied to the polyacrylamide gel.

Having identified J chain in the tissue culture-adapted MOPC 315 myeloma cell line, we then examined a variety of other tumour lines using similar methodology. The results are summarised in Table 1. Not unexpectedly, J chain was detected in cells secreting polymeric IgM (MOPC 104E) and IgA (MOPC 315 and 5647). More interesting was the finding of comparable amounts of J chain within myeloma cells secreting IgG₁ (MOPC 21 and MOPC 70E) and IgG_{2a} (Adj PC5 and 5563). In all cell lysates where a positive identification of J chain was made, there was no characteristic peak on polyacrylamide gel when the rabbit antiserum was absorbed with purified mouse J chain before the formation of the antibody-antigen precipitates with goat anti-rabbit immunoglobulin. We are therefore confident of our identification of J chain in IgG-secreting plasma cells. Relatively less J chain was present in a myeloma which secretes half molecules (HL) of an IgA (MOPC 47A) containing heavy chains of shorter than normal length^{18,19}. No J chain could be detected in a tumour line which secretes only monomer (H_2L_2) species of IgA (MPC1). This is further evidence that the peak identified on alkaline-urea polyacrylamide gels is authentic J chain, because if this component were a polypeptide which fortuitously cross-reacted with J chain, its presence would be predicted in all myelomas.

The finding of J chain in IgG-secreting myeloma cells raises the question of whether J chain is restricted to cells of lymphoid origin. We therefore labelled a permanent cell line of mouse L cells and looked for J chain, as detailed in the

TABLE 1 Presence of J chain in mouse myeloma cells.

Tumour	Product	J chain (% total radioactivity $\times 10^{-2}$)
MOPC 104E	IgM, free L	3.29
MOPC 315	IgA (monomer and polymer)	2.81
5647	IgA (monomer and polymer)	1.03
MPC 1	IgA (monomer only)	Not detected
MOPC 47A	IgA (HL half molecule)	0.26
MOPC 21	IgG ₁	1.35
MOPC 70E	Free L (K), traces of IgG ₁	3.17
Adj PC5	IgG _{2a}	2.45
5563	IgG _{2a}	1.32

Myeloma cell suspensions were prepared from solid tumours, incubated for 2 h with radioactive leucine and cell lysates were prepared as indicated in the legend to Fig. 1. Total radioactivity in protein was measured by precipitation with 10% (w/v) trichloroacetic acid. Samples containing 2×10^7 – 6×10^7 c.p.m. were reacted with the rabbit antimouse J chain antiserum and goat anti-rabbit immunoglobulin, and the precipitates were analysed on alkaline-urea-polyacrylamide gels as described in the legend to Fig. 1. The radioactivity in J chain was obtained by summing the counts found in the position on the gel characteristic for J chain. This amount is expressed as % of the total trichloroacetic acid-precipitable radioactivity in the cell lysate. Verification of identity was obtained in all cases by electrophoresis of precipitates made using a sample of rabbit anti-mouse J chain previously absorbed with purified mouse J chain (see text and legend to Fig. 1).

legend to Fig. 1. J chain could not be detected in radioactive lysate samples containing up to 6×10^7 c.p.m. Although more non-lymphoid cells need to be examined, nonetheless the tentative conclusion is that J chain is not a ubiquitous cellular constituent and may be confined to cells of lymphoid origin.

What is the fate of J chain synthesised by IgG-secreting cells? One possibility is intracellular degradation and another secretion. The latter is more easily tested. Myeloma cells secreting IgM (MOPC 104E), IgA (MOPC 315), IgG₁ (MOPC 21) and IgG_{2a} (5563 and Adj PC5) were incubated with radioactive leucine and allowed to secrete for a period of 5 h. Culture supernatants containing 2×10^7 c.p.m. in trichloroacetic acid-precipitable radioactivity were tested for the presence of free J chain using the specific rabbit antiserum as already described. In all cases tested free J chain was absent. This makes it more likely that it is degraded following synthesis by IgG-secreting cells. This would be compatible with the absence of covalent and non-covalent interaction between intracellular immunoglobulin and J chain^{7,10}, and the fact that only at the subcellular site where polymerisation takes place is J chain incorporated into polymer molecules^{10,11}. As polymerisation takes place just before, or at the time of, secretion¹⁻⁷, it is reasonable to suggest that newly synthesised J chain follows the same intracellular route irrespective of the class of immunoglobulin secreted. If J chain is degraded in IgG-secreting cells, this probably occurs at the same subcellular site as polymerisation.

The failure of the IgA-secreting tumour line MPC1 to secrete polymeric molecules is not entirely attributable to the absence of J chain. The reason for this assertion is that secreted IgA monomers of MOPC 315 can be polymerised *in vitro* when J chain and a disulphide interchange enzyme are supplied¹⁶. In identical conditions, the secreted monomeric IgA of MPC1 is not polymerised (R.M.E.P., and E. Della Corte, unpublished). A likely explanation would be a structural alteration in the heavy chain, perhaps at the site involved in linkage to the J chain. The secreted HL subunits of MOPC 47A were also unable to form polymers *in vitro* when supplied with J chain and the disulphide interchange enzyme (R.M.E.P., and E. Della Corte, unpublished). Extensive deletions are known to be present in the heavy chain of this tumour line^{18,19}. The presence or absence of intracellular J chain, together with the potential for secreted immunoglobulin to be polymerised *in vitro*, should form the basis of a valuable approach for analysis of myeloma variants secreting IgA and IgM.

The MOPC 70E tumour line secretes traces of IgG₁, but the major secretory product of the cell is light chain. In this situation of suppressed heavy chain synthesis, however, intracellular J chain was demonstrated, indicating that the genes for heavy chains and J chains are not coordinately controlled.

What is the significance of J chain present in myeloma cells secreting IgG? While other interpretations must be considered, for example that the cells studied are neoplastic, it is possible to speculate that intracellular J chain in IgG-secreting cells represents a relic from previous commitment to IgM synthesis, which would also allow the same progenitor cell line to switch from IgG to IgA synthesis. Such a developmental succession of immunoglobulin class expression has already been postulated¹³.

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Expression of polyoma-induced antigens in low malignant hybrids derived from fusion of a polyoma-induced tumour with a fibroblast line

HARRIS *et al.*¹ have shown that hybrid cells (A9/SEWA) derived from fusion of cells of polyoma-induced sarcoma (SEWA) and an aneuploid long-established fibroblast line (of L origin), A9, had low tumorigenicity (Table 1)². They have also shown³ that the polyoma-induced transplantation antigen (PTA) was present in low tumorigenic hybrids of SEWA/T6 T6. To investigate the relationship between malignancy and cell surface modifications, we analysed, in this system, the polyoma-specific surface antigen revealed by immunofluorescence⁴ concurrently with other characteristics of these cells, such as karyotype, transplantability and the presence of T antigen.

Antigen T was demonstrated by immunofluorescence on fixed cells as previously described^{5,6}. Two positive antisera were used. The counts were done on 10^3 cells for three independent experiments. The parental SEWA cells and the A9/SEWA hybrid were both strongly positive. The parental A9 strain was negative (Table 2).

Surface antigen was studied on living cells using the technique we have previously described⁴. Five hundred cells were counted and two repeat counts performed for each experiment. The antisera were produced in A.S.W. mice injected with 10^7 PFU polyoma virus once a week for 3 weeks. After an interval of 1 week, the mice were challenged with 10^3 SEWA cells injected intraperitoneally. The sera collected from the tumour-free mice, 2 weeks later, were tested by immunofluorescence using SEWA cells as the positive control and A9 and A.S.W. mouse kidney cells as negative controls. Positive results were also obtained when these sera were tested against cells of a cell line (C3H Py Broc.) that had been

TABLE 1 Growth of cells *in vivo**

Cell type	Genotype of host	Unirradiated animals		Irradiated animals	
		No. takes (Total no. animals with progressive tumours/total no. inoculated)	% takes	No. takes (Total no. animals with progressive tumours/total no. inoculated)	% takes
A9	C3H			3/25	12
SEWA/A9	C3HxA.SW F ₁			8/25	32
SEWA†	A.SW	4/4	(100)	17/17	100
SEWA†	A.SW	45/61	73	93/98	93

* 5×10^4 to 2×10^6 cells were injected subcutaneously in syngeneic newborn animals

† Tumour cells adapted to growth *in vitro*

transformed *in vitro* by polyoma virus. The results, summarised in Table 2, show that the hybrid does carry the polyoma-specific membrane antigen in very similar proportions (about 80%) whatever the passage level.

at 4° C. The serum was then retested with the target cells (SEWA cells in ascitic form). Absorption with 5×10^7 SEWA cells in ascitic form ml⁻¹ and 2.5×10^7 A9/SEWA cells ml⁻¹ was necessary to obtain identical extinctions (Ta-

TABLE 2 Polyoma antigens

Cells	A9	SEWA	A9/SEWA 8th passage*	A9/SEWA about 10th passage*	A9/SEWA about 40th passage*	A9/SEWA 43rd passage*
T antigen % of positive cells	0	60 ± 10		30 ± 10	20 ± 10	
Polyoma-specific surface antigen 11% of positive cells	0.4	88 ± 5	82 ± 5			88 ± 5

* Average results of four experiments done on subcultures about tenth or fortieth serial passage after the receipt of the hybrid at Marseilles.

Experiments with antiserum absorption gave results which did not differ significantly between the parent lines and the hybrids. The following technique was used in these antiserum absorption experiments. The limiting dilution (1/320) of the anti S-polyoma serum was first determined using a

TABLE 3 Absorption of antiserum by ascitic SEWA and A9/SEWA cells

Cell line	Number of cells ($\times 10^7$) used for absorption	% positive cells
SEWA	0.5	80
	1	80
	2.5	30
	5	5
	10	5
A9/SEWA	0.5	80
	1	20
	2.5	5
	5	5
	10	5

Anti S-Polyoma serum at 1/320 dilution was absorbed by contact with the cells for 1 h at 37°C and overnight at 4°C. The serum was then retested on the target cells (SEWA and A9/SEWA).

1/10 dilution of fluorescein-labelled anti-mouse immunoglobulin. The absorption was then carried out by contact of the serum at 1/320 with the cells for 1 h at 37° C and overnight

TABLE 4 Surface histocompatibility antigens measured by immunofluorescence

Cell antigens	A9	SEWA	A9/SEWA
H-2k*	45%	2%	35%
H-2s*	4%	30%	23%

* Once a week A.SW mice were injected intraperitoneally with 10^6 C3H mouse spleen cells for 3 consecutive weeks. These spleen cells were prepared for injection as follows: the spleen was removed and the cells scraped off with a file. The resulting cell suspension, which contained many red blood cells was first washed in PBS free of Ca⁺⁺ and Mg⁺⁺ by centrifuging at 200g for 5 min. After treatment by hypotonic shock, the cells were collected by a second centrifugation. The same operation was performed with C3H mice injected with spleen cells of A.SW mice; sera were used at 1/30 dilution.

TABLE 5 Immunogenicity

A.SW pretreated with	Total N° of inoculated cells	Takes in A.SW mice 20 d after challenge
10 ⁶ PFU polyoma	—	2/11
A9/SEWA*	3×10^6	3/22
UV irradiated SEWA (3,000 erg mm ⁻²)	3×10^6	2/6
A9	3×10^6	5/5
Control	—	14/15

* Average results of three experiments done on subcultures about thirtieth and sixtieth serial passage after receipt of hybrid at Marseilles.

Growth in 2 month old A.SW mice of SEWA in ascitic form after inoculation of various agents once a week for 3 week interval of 2 week and then a challenge I/P injection of 1×10^8 SEWA cells.

ble 3). When the cell size and the fact that about 30% of the cells in the ascitic form of the SEWA tumour are not tumour cells (for example the macrophages and endothelial cells) are considered, this difference cannot be regarded as significant.

The results of testing for H-2 antigens are shown in Table 4 and the ability of various test cells to induce a rejection reaction of TATA-positive SEWA cells in syngeneic A.SW mice are shown in Table 5.

The karyotype study, which mainly took into account the evolution of the mode and the presence of markers with respect to the passage level of the cell lines, was carried out both for the hybrid and parental strains, and is summarised by the histogram in Fig. 1.

The presence of these antigens indicates that the viral genome is expressed in the hybrid cells. We have demonstrated previously that at least half of the polyoma genome necessary for replication is involved in the coding for the expression of T and S antigen^{7,8} and one fifth for the expression of TATA. It is also clear that the introduction of a fibroblast genome represses malignant behaviour, as shown previously⁹ but does not interfere with the polyoma-specific antigen expression of the transformed cells. In view of the fact that only certain cells can be transformed by polyoma virus, the genetic or the epigenetic constitution of the target cell is obviously decisive for its competence to behave as

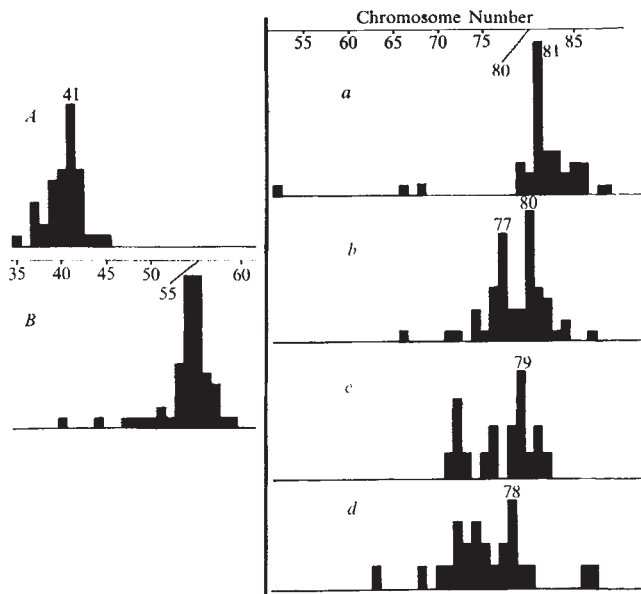


FIG. 1 Karyotype analysis of hybrid and parental strains. A, SEWA; B, A19; a, A9/SEWA hybrid, third passage; b, twenty-eighth passage; c, seventieth passage; d, 118th passage.

a malignant cell after the covalent integration of the viral genome. The role of the cellular genome is also suggested by the experiments on reversion¹⁰, showing that rearrangement of the genetic material may suppress the expression of transformed and/or neoplastic properties, in spite of the continued presence of the viral genome. The same conclusion is reinforced by our findings on hybrid cells. Obviously the genome of at least certain fibroblasts will inhibit tumour growth, in spite of the continued presence of the virally determined antigens. Although these antigens may be necessary for the malignant behaviour of the polyoma transformed cell, with the appropriate genetic or epigenetic constitution, it will follow from this reasoning that this is not necessarily sufficient and other modifying factors may play a decisive role for the realisation of the virally determined neoplastic potential.

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Lipopolysaccharides inhibit lymphosarcoma cells of bone marrow origin

BACTERIAL lipopolysaccharides (LPS) stimulate DNA synthesis specifically in B (thymus-independent) lymphocytes¹⁻⁴, increase immunoglobulin synthesis and secretion in B cells⁵, and stimulate the immune response of antibody production to various antigens in the apparent absence of T (thymus-dependent) lymphocytes^{4,6-8}. Recently a mouse B lymphosarcoma, PU-5-1, has been described which has surface immunoglobulin⁹, and produces a small amount of immunoglobulin (R. Asofsky, unpublished). Cells of this tumour were adapted to culture and tested for the LPS stimulation of immunoglobulin secretion. Surprisingly, growth of the tumour cells was inhibited by LPS, although there was no effect on mouse myelomas or T lymphomas. Inhibition also occurred with an Abelson leukaemia virus induced tumour believed to represent a B lymphoma¹⁰ or immature myelomonocytic neoplasm¹¹. No effect was observed on the growth rate of a number of human B cell lines tested. LPS is not cytotoxic for the tumour cells: growth resumes when it is removed. Incorporation of precursors into DNA is inhibited before RNA and protein synthesis is affected. The active com-

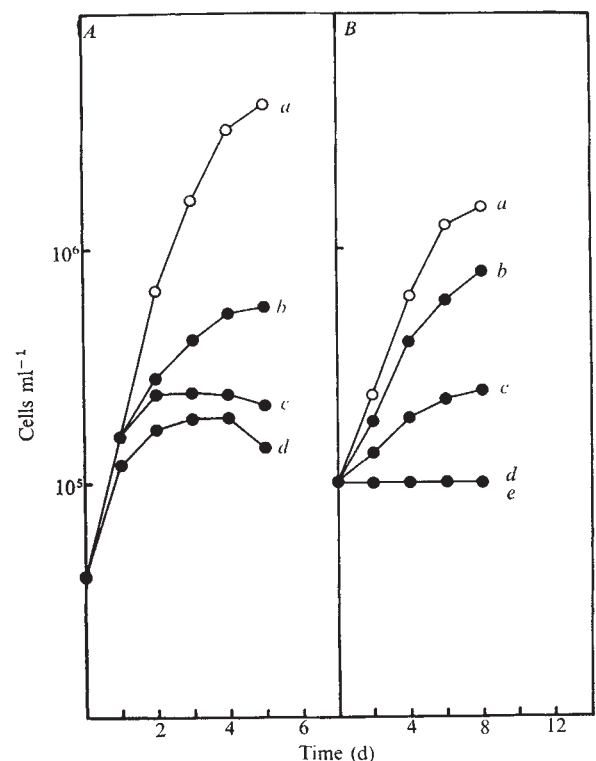


FIG. 1 Growth inhibition by LPS. A, RAW 8 cell line was adapted to culture from the second transplanted generation of a BALB/c tumour originating from infection of spleen cells *in vitro* with Abelson leukaemia virus³⁰ (in preparation). The cells grow as round floating cells in Dulbecco modified Eagle's medium containing 10% foetal calf serum in Petri dishes¹⁶. LPS (*S. typhosa* W 0901, Difco Laboratories, Detroit) was dissolved in phosphate buffer, pH 8.0, at 10 mg ml⁻¹, boiled for 60 min, and stored frozen until use. Cultures were inoculated at 0.4×10^5 ml⁻¹ with a, 0; b, 1; c, 5 or, d, 50 μ g ml⁻¹ as shown. Viable cell counts were made daily. B, PU-5-1 is a spontaneous lymphosarcoma from a germ-free BALB/c mouse with immunoglobulin μ and κ determinants on its surface⁹ (obtained from R. Asofsky, N.I.H., Bethesda, Maryland). The tumour was adapted to culture at generation 35 and grown in RPMI-1640 medium with 10% horse serum as above. Cultures were initiated at 1×10^5 cells ml⁻¹ with a, 0; b, 1; c, 5; d, 20 or e, 50 μ g ml⁻¹ LPS as shown.

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TABLE 1 Cell lines tested for LPS inhibition

Murine		Primate	
Sensitive		Sensitive	
PU-5-1.8	B lymphoma	None tested	
RAW 8.3	Lymphosarcoma		
Resistant		Resistant	
S49.1	T lymphoma		
WEHI 22.1	"	MLC	Marmoset
EL4	"	T cell line	
R1	"		
C1	Myeloma	Human B cell lines	
P3	"		
J558	"		
MOPC 315	"	RAJI	Burkitt's lymphoma
S117	"	DAUDI	"
GM-86	Erythroleukaemia	WIL2	Normal
P815	Mastocytoma	8866	
C1498	Myeloid leukaemia	UM-37	

Cell lines were tested for growth inhibition by LPS as in Fig. 1. Cultures of cell lines listed as Resistant contained more than 50% of the number of cells in control cultures when incubated 4–8 d with $100 \mu\text{g ml}^{-1}$ LPS. P815-X2 was obtained in culture from D. Grausz, University of California at San Diego; WEHI-22.1 from A. Harris, Walter and Eliza Hall Institute, Melbourne; GM-86 from Institute for Medical Research, Camden, New Jersey. The other mouse cell lines are listed in ref. 16. Marmoset and human cell lines were obtained through the courtesy of R. Lerner²⁸, Scripps Clinic and Research Foundation, La Jolla, California; G. Moore¹⁷, Denver General Hospital, Denver; A. Bloom²⁹, University of Michigan, Ann Arbor; and G. Klein, Institute of Tumor Biology, Stockholm. UM-37 was derived from a patient with myeloma. The primate lines were grown in RPMI-1640 medium containing 10% foetal calf serum in Petri dishes.

ponent of LPS seems to be the lipid A, as shown for its pyrogenic¹², mitogenic^{8,13–15}, and adjuvant^{8,13} properties.

At $1\text{--}50 \mu\text{g ml}^{-1}$, LPS from *Salmonella typhosa* inhibited the growth of RAW 8 cells (Fig. 1), a lymphosarcoma of a BALB/c mouse derived from *in vitro* infection of spleen cells with Abelson leukaemia virus. Cell growth continued normally for one or two doublings, and then was completely inhibited in the presence of $5 \mu\text{g ml}^{-1}$ LPS. Growth of PU-5-1, a spontaneous BALB/c B-type lymphoma⁹ was inhibited by LPS at $5\text{--}50 \mu\text{g ml}^{-1}$ without a delay (Fig. 1). LPS from *E. coli* (W 055:B5 and 0111:B4, Difco) gave similar results. Lipid A derived from LPS by mild acid hydrolysis also inhibited RAW 8 and PU-5-1 cell growth at $5\text{--}20 \mu\text{g ml}^{-1}$ (not shown). These concentrations of LPS and lipid A which are inhibitory to the tumour cells were previously shown to be optimally mitogenic for normal spleen B lymphocytes^{4,8}.

Cell lines which are not sensitive to LPS inhibition of growth are listed in Table 1. Among murine tumours these include, θ^+ T lymphomas, myelomas, a myeloid leukaemia, an erythroid leukaemia, and a mastocytoma. Some cell lines show a partial inhibition of DNA synthesis 24 h after LPS addition, such as the T lymphoma S49¹⁶, but DNA synthesis resumes and overall growth does not seem to be affected. A number of human lymphoid cell lines with surface immunoglobulin, considered to be B cells, and a marmoset T cell line are also unaffected by LPS (Table 1). A human myeloma line¹⁷, RPMI 8226, is partially inhibited by LPS.

Experiments were performed to determine the sequence of events during the LPS inhibition of synthesis of macromolecules. Figure 2 shows that $20 \mu\text{g ml}^{-1}$ LPS causes a rapid inhibition of incorporation of ^3H -thymidine into DNA in RAW 8 cells, followed by a decrease in apparent RNA synthesis, both approaching 30–40% of control levels by 24 h. As two doublings occur before growth stops, one possibility is that an early event involves the LPS inhibition of transport of nucleic acid precursors rather than direct inhibition of DNA synthesis. Protein synthesis, as measured by the uptake of an essential amino acid, continues unaffected for more than 24 h before showing impairment. PU-5-1 cells also

exhibit a rapid decline of incorporation into DNA, but not into RNA or protein (Fig. 2).

As cells remained viable for up to 5 d during LPS inhibition of growth, reversal of the LPS effect was tested. Both RAW 8 and PU-5-1 cells, cultured in the presence of $20 \mu\text{g ml}^{-1}$ LPS for 2 d, resumed DNA synthesis and multiplication when washed and recultured without LPS (not shown).

A screening was made of drugs with biological effects similar to LPS. BCG is an immunological adjuvant stimulating the reticuloendothelial system¹⁸, and synthetic polyribonucleotides have toxic and adjuvant properties like LPS¹⁹, but BCG (at 4×10^5 viable bacteria ml^{-1} ; Eli Lilly), poly(I)·poly(C) ($100 \mu\text{g ml}^{-1}$; P L Biochemicals) and poly(A)·poly(U) ($100 \mu\text{g ml}^{-1}$; Miles Laboratories) did not inhibit growth of RAW 8 cells. Some thymus-independent antigens such as dextran (molecular weight 500,000, Pharmacia) and polyvinyl pyrrolidone (molecular weight 160,000, Baker; molecular weight 360,000, Sigma) are reported to be mitogenic specifically for B lymphocytes²⁰, as is LPS, but do not affect RAW 8 growth at 1 mg ml^{-1} . Dextran and zymosan, like LPS, activate complement components²¹, which bind to B lymphocytes. Zymosan caused only moderate inhibition of RAW 8 growth at 1 mg ml^{-1} (Sigma, 50% of control cell number at day 4 as in Fig. 1) and the LPS inhibition occurred equally well in fresh or heat-inactivated foetal calf serum, indicating that complement is not involved.

A variety of microbiological products and synthetic polynucleotides are under investigation for nonspecific stimulation of host immunological resistance to tumours¹⁸. LPS and endotoxins are reported to have inhibitory effects on tumours *in vivo*²², and this has been correlated with the local Schwartzman reaction and haemorrhagic necrosis²³. With some tumours, the endotoxin effect may be mediated by factors released by the host rather than a direct action on the tumour²³. The experiments described here suggest that certain lymphosarcomas may be susceptible also to direct inhibition by LPS. In preliminary experiments, mice given RAW 8 and PU-5-1 tumour cells, preincubated 1 h with LPS, or mice treated with LPS injections 2 d after inoculation, survived significantly longer than controls. LPS treatment did not increase survival of mice with other types of tumours.

Not all tumours derived from B lymphocytes are inhibited by LPS. Mouse myelomas and human B lymphoid lines are

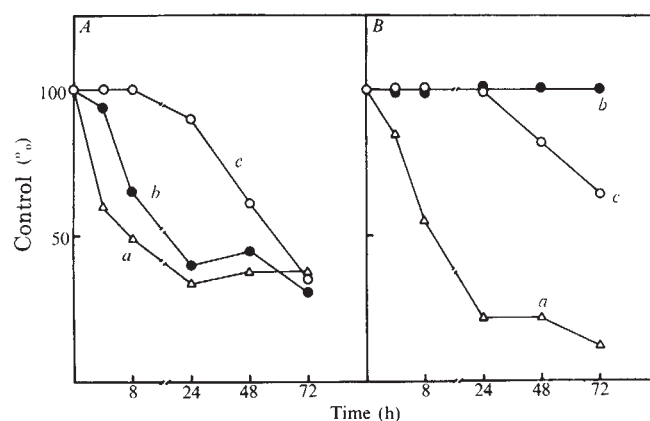


FIG. 2 Effect of LPS on incorporation into DNA, RNA and protein. Two ml cultures of A, RAW 8 or B, PU-5-1 were inoculated with or without $20 \mu\text{g ml}^{-1}$ LPS as in Fig. 1. At the times shown, duplicate dishes were incubated 1 h with $1 \mu\text{Ci } ^3\text{H}$ -thymidine (50 Ci mmole^{-1}), $1 \mu\text{Ci } ^3\text{H}$ -uridine (25 Ci mmole^{-1}), or $10 \mu\text{Ci } ^3\text{H}$ -leucine (medium contains 0.8 mM leucine) to measure incorporation into, a, DNA; b, RNA or, c, protein, respectively. At the end of 1 h, the cells were collected and TCA-precipitable radioactivity determined. Each point is the mean of measurements from duplicate cultures which agreed within 10%. Results are shown as incorporation in the presence of LPS as a % of control cultures.

resistant (Table 1). There may be some types of human lymphosarcomas, however, which resemble RAW 8 and PU-5-1 in LPS sensitivity. Human peripheral blood lymphocytes²⁴ and spleen cells (P.R., and I.N., unpublished) do not respond to the mitogenic effect of LPS. LPS is not mitogenic or toxic to the mouse strain C3H/HeJ (ref. 25). Experiments are in progress to induce Abelson leukaemia virus tumours in these mice to see which biological activities are correlated with lymphosarcoma inhibition.

The inhibition of B lymphomas by LPS which is a mitogen for normal B lymphocytes is curious. A similar paradox was seen in the toxicity of PHA, a T lymphocyte mitogen, for T lymphomas but not other tumours^{16,26}. The stimulating signals from the mitogens on the already dividing tumour cells may result in a push to a differentiated end cell type incapable of further division. Further proof is needed that the LPS-sensitive cells described here are tumours of B lymphocytes rather than of a form of monocyte as LPS is reported to have inhibitory effects on macrophages or phagocytic mononuclear cells²⁷.

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Complement-mediated mixed aggregation of murine spleen cells

RECEPTORS on the surface of B cells for the fixed C3 component of complement have previously been studied by investigating the adherence to lymphocytes of complement-coated immune complexes and their function interpreted in the light of this reaction¹⁻¹⁰. Other nucleated cells, however, notably macrophages, in the secondary lymphoid organs, also have receptors for fixed C3 (refs 3 and 4). It is therefore possible that alexinated complexes might mediate mixed adherence of different cell types which could have more significant functional consequences for complement receptor lymphocytes than just direct binding to their C3 receptors.

In vivo and *in vitro* studies indicate that C3 plays a part in the induction of thymus-dependent humoral antibody formation, but not in the response of B cells either to mitogens or thymus-independent antigens (refs 11, 12, 12a and unpublished results of M.B.P.). I have suggested on the basis of this evidence that C3 fixed on antigen-containing complexes might, as a consequence of its interaction with cell surface C3 receptors, enhance or facilitate T-dependent presentation of antigen to B cells at the macrophage surface (refs 11 and 13 and M.B.P., unpublished). Here I confirm the feasibility of this model and demonstrate the complement-mediated mixed adherence of spleen cells which results from binding of fixed C3 to cell surface C3 receptors.

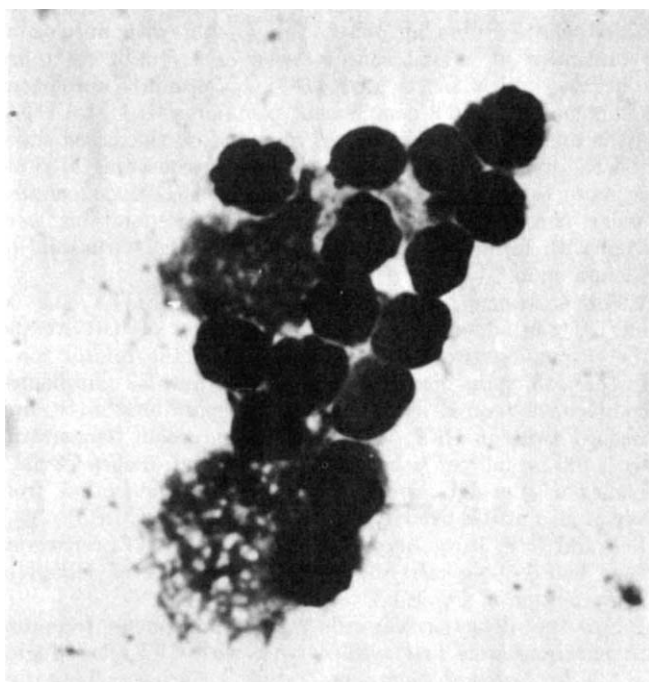


FIG. 1 Complement-mediated spleen cell aggregation. A dissociated spleen cell suspension from a mouse which had received 0.2 ml of Freund's complete adjuvant by intraperitoneal injection 3 weeks previously was rotated at 37°C for 45 min with fresh autologous serum (1/5 in CFT). The appearance of a typical cell clump in a fixed preparation obtained at the end of incubation, stained with Giemsa stain is shown. Cells with morphology characteristic of small lymphocytes, macrophages and blast cells were present. (Magnification $\times 1,550$).

TABLE 1 Complement-mediated spleen cell aggregation*

Lymphocyte suspension†	Complement‡	Other treatment	% Clumps ¶	Size of clumps**
Normal spleen	NMS	—	15	15–50
Normal spleen	NMS/inact	—	2	3–5
Normal spleen	NMS/EDTA	—	0.2	3–5
Adjuvant spleen	NMS	—	16	>50
Adjuvant spleen	NMS/inact	—	3	5–15
Adjuvant spleen	NMS/EDTA	—	1	3–5
Adjuvant spleen	NMS	CFT §	14	15–50
Adjuvant spleen	NMS	EDTA §	0.4	3–5
Adjuvant spleen	NMS	IgG ₁	14	15–50
Adjuvant spleen	NMS	anti-C3	1	3–5
Normal thymus	NMS	—	3	3–5
Normal thymus	NMS/inact	—	3	3–5

* Lymphocyte suspensions were rotated for 45 min at 37°C with active or inactive complement.

† Cell suspensions of spleens from normal mice, mice which had received an intraperitoneal injection of 0.1 ml of complete Freund's adjuvant during the preceding month, or of normal adult thymus cells.

‡ NMS, Fresh autologous normal mouse serum; NMS/inact, same serum heated to 56°C for 30 min; NMS/EDTA, same serum containing 0.01 M EDTA.

§ CFT or EDTA to 0.01 M added to cell suspensions in complement after 45 min incubation and rotation continued for further 15 min. || Cell suspensions incubated for 45 min with complement were washed in CFT and then incubated at room temperature with either eluted anti-C3 antibodies or the IgG₁ fraction of anti-mouse C3.

¶ Number of clumps of >3 cells among 500 free nucleated cells expressed as a %.

** Approximate mean of the number of cells in the clumps.

Lymphoid cells were prepared by crushing the freshly removed spleens or thymuses of adult BALB/c mice with a silicone rubber bung and suspending the cells in complement fixation test diluent (CFT) (veronal buffered physiological saline containing Ca²⁺, Mg²⁺, gelatin 0.1 % w/v and 0.015 M sodium azide). Debris and clumps were removed by rapid filtration through separate 2.0 ml cotton wool columns for individual organs; the resulting suspensions after two washes in CFT at 4° C consisted exclusively of single cells. Each mouse was exsanguinated, the serum separated within 12 h at room temperature and kept at 4° C until use on the same day. For the production of complement-mediated cell clumps, lymphoid cell suspensions (5×10^6 – 15×10^6 cells ml⁻¹) in CFT were rotated (20 r.p.m.) in sealed plastic tubes at 37° C for 45 min with autologous complement at a final concentration of 1:5. Control tubes contained either inactivated (56° C, 30 min), autologous complement or fresh complement containing 0.01 M EDTA. After rotation, the mixture was mounted on siliconised slides and the number of nucleated cell clumps, consisting of three or more cells, amongst 500 free nucleated cells was counted under phase contrast. Dried smears of preparations were fixed with formal-saline and stained with May-Grunwald or Giemsa stain.

For treatment of formed clumps with EDTA, 0.2 M EDTA was added to a final concentration of 0.01 M and the suspensions were then reincubated on the rotator for a further 15 min. For treatment with anti-C3 antibodies, complement-treated lymphoid cell preparations were first washed twice in CFT, then incubated at room temperature with 100 µg ml⁻¹ of either isolated sheep anti-mouse C3 antibodies or the IgG₁ fraction of the same antiserum from which all anti-C3 activity had been absorbed (M.B.P., A. J. Bell and I. F. Rowe, unpublished). The anti-C3 preparation used had 170 µg ml⁻¹ of protein and a titre of 1:256 for agglutination of 1% EAC.

For the detection of cells with complement receptors, preparations were first washed twice with CFT, centrifuging at 90g for 5 min at room temperature. Indicator cells bearing fixed murine C3 were prepared by sensitising sheep erythrocytes (E) with 6 MHD of IgM rabbit anti-sheep erythrocyte antibody (A) and then alexinating them by incubation (15 min, 37° C) of 1% EA with 1:10 fresh normal mouse complement (C). The EAC were washed twice in CFT before use. Lymphoid cells (20 µl at approximately 2×10^6 cells ml⁻¹) and 0.5% EAC were mixed in siliconised glass Rh rubes and centrifuged at 90g for 5 min at 4° C. The cell pellet was then gently but thoroughly resuspended with a Pasteur

pipette and mounted in 0.5% toluidine blue on siliconised slides. Nucleated cells with three or more adherent red cells were scored as rosettes and the proportion of these among at least 200 nucleated cells was counted for each tube. In some experiments, parallel assays in which EDTA to a final concentration of 0.02 M was present were included. Four replicates of each assay were performed, and the % of rosette-forming cells was corrected for the number of rosettes formed by EA instead of EAC.

Incubation of a suspension of normal mouse spleen cells with fresh autologous serum induced the formation of clumps of cells (Table 1). The size and number of clumps were greater when spleen cells were used from mice which had received an intraperitoneal injection of complete Freund's adjuvant in the preceding 2–4 weeks. If the serum was inactivated by heating at 56° C for 30 min, or if it was made to 0.01 M EDTA, no cell clumps formed, nor did they if cell suspensions were incubated in the absence of serum. The formation of clumps was optimal after about 45 min of incubation. Continued rotation at 37° C was associated with progressive dissolution of the aggregates; they were, however, more stable on standing at room temperature and persisted unchanged for up to 48 h at 4° C. The clumps consisted of a heterogeneous population of cells which included small lymphocytes, macrophages, occasional polymorphs and, when stimulated spleens were used, occasional blast cells (Fig. 1). Thymus cell suspensions did not aggregate when incubated with complement under the same conditions.

Incubation of the source of complement with the C3-cleaving protein (CoF) of cobra venom (refs 14 and 15 and M.B.P., unpublished) so as to induce complete conversion of C3 on antigen-antibody crossed electrophoresis rendered it inactive in the production of cell clumps. Confirmation of specific dependence of the phenomenon on C3 was obtained by incubating washed clumps at room temperature with specific eluted anti-mouse C3 antibodies; this resulted in almost complete dissolution of the aggregates (Table 1). Addition of EDTA to 0.01 M to spleen cell suspensions clumped by complement resulted in rapid and complete dissociation of clumps (Table 1), suggesting that macrophages, (to which, in the mouse, the adherence of fixed C3 requires magnesium ions³), are involved.

If complement-clumped spleen cell preparations were washed and then rosetted with EAC in the presence of divalent cations, numerous red cells adhered to the clumps and the overall proportion of rosettes on mononuclear cells was diminished by comparison with the initial spleen cell suspension untreated with complement (Table 1). When the

TABLE 2 Effect of preincubation of spleen cells with complement on complement rosettes

Treatment of spleen cells*	% Complement rosettes†		Clumps‡	
	CFT	EDTA	CFT	EDTA
Nil	36.3 ± 5.5	20.7 ± 2.1	—	—
NMS	23.7 ± 3.5	12.7 ± 3.1	+	—
NMS then EDTA	23.7 ± 2.5	12.3 ± 2.1	+	—
NMS/inact	45.0 ± 4.4	23.0 ± 2.6	—	—

* Spleen cell suspensions were rotated at 37°C for 45 min with CFT, autologous fresh serum or heat inactivated autologous fresh serum, before washing in CFT. Some cells incubated with complement were also washed with EDTA.

† Mean ± s.d. of four replicates of cells rosetting with EAC in the presence or absence of Mg²⁺.

‡ The presence or absence of appreciable clumping of spleen cells. When clumps were present many indicator cells adhered to them.

clumped preparations were rosetted in the presence of 0.01 M EDTA the clumps dissociated, but there was an even greater reduction in the proportion of rosettes formed. The numbers of rosettes formed by clumped preparations which had been dissociated by EDTA, before rosetting in the presence and absence of divalent cations, showed a similar degree of reduction in comparison with the values obtained with the starting preparation. In contrast, treatment of spleen cells with inactivated mouse serum had no effect on the subsequent capacity of the cells to form rosettes with EAC (Table 2). These results suggest first, that after incubation of spleen cells with complement, cells with C3 receptors are included in the clumps; second, that as most of the rosettes which do form among the complement-treated cells are inhibitable by EDTA, and are therefore presumably on macrophages, the lymphocyte C3 receptors are apparently 'blocked' following incubation with complement and do not bind EAC¹⁶.

Spontaneous dissociation of the complement-mediated clumps in the continued presence of the complement source at 37° C may have resulted, at least in part, from alternative pathway-mediated dissociation of fixed C3 from B cells¹⁷. This is probably due to generation of fluid-phase C3 fragments which have been shown in both a homologous human system (ref. 11 and M.B.P., and A. E. Butterworth, unpublished) and a mixed mouse-guinea pig system¹⁸ to compete with fixed C3 for the C3 receptors. The fact that C3-dependent mixed cell adherence described here does reverse spontaneously provides further support for the model which has been proposed for a possible role of this process in cell cooperation (ref. 11 and M.B.P., unpublished).

Complement-mediated C3-dependent aggregation of the different cell types in spleen cell suspensions which bear receptors for fixed C3 is of interest for several reasons. First, it illustrates the feasibility of an *in vivo* role for C3 in mediating the approximation of different cell types, particularly B cells and macrophages, which may be relevant to the mechanisms of cell cooperation in the induction of antibody production. Second, it provides evidence for the existence in normal spleens, and possibly to a greater extent in the spleens of mice stimulated by antigen, of a cell-associated mechanisms or mechanisms capable of fixing C3.

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Role of heterochromatin in the control of cell cycle duration

RECENTLY Bennett has demonstrated that most annual plants have less nuclear DNA than perennial plants¹. Since there is a positive correlation between DNA content and minimum duration of the mitotic cell cycle², Bennett suggested that low DNA content and short cycles might be factors determining the generation time of short-lived plants. These assumptions also fit the general findings that DNA contents have increased strikingly during general evolution³ but have decreased during specialisation⁴⁻⁶.

Although the progression from DNA-rich conservative species to DNA-poor advanced species could also be demonstrated in some perennial Anthemideae (Asteraceae, Dicotyledones)⁷, several annuals have been found in this subfamily which have significantly more DNA than perennials of the same genera⁸. In spite of this, the annual species develop much more rapidly than the perennials. Therefore, a factor unconnected with nuclear DNA content might be expected to play a role in the control of cell cycle and generation times in those species.

I have studied the relationship between nuclear DNA content, percentage of heterochromatin, and cell cycle duration in the genera *Anacyclus*, *Anthemis*, and *Artemisia*. I have analysed three perennial species, three annual species with a higher DNA content, and two annual species with a lower DNA content than the related perennials. I found evidence that the amount of heterochromatin per genome is related to the reduction of cell cycle times in annual plants with large DNA contents.

Seedlings were grown in controlled environment chambers at 22° C and 15 h light; root tips were fixed in ethanol:acetic acid (3:1) and Feulgen-stained for DNA measurement by cytophotometry. The roots were immersed in a solution of 10 μ Ci ml⁻¹ 6-³H-thymidine specific activity 26 C mmol⁻¹ at 22° C for 1 h and fixed every other hour for 2 d. Labelled metaphases were counted after an exposure time of 3 d.

The duration of the mitotic cell cycle can be estimated for every species for which the DNA content is known,

TABLE 1 Nuclear DNA contents, heterochromatin content and duration of cell cycle in root tips of annual and perennial *Anthemideae*

Species	Habit	DNA (10 ⁻¹² g per 2C nucleus)	Heterochromatin-DNA (% of total DNA)	Cell cycle time (h)		
				Expected	Actual	Difference
<i>Anacyclus depressus</i> Bail.	Perennial	12.42	1.62	14.3	15.9	+1.6
— <i>clavatus</i> (Desf.) Pers.	Annual	10.48	8.55	13.0	11.0	-2.0
— <i>radiatus</i> Lois.	Annual	16.92	6.84	16.2	13.6	-2.6
<i>Anthemis tinctoria</i> L.	Perennial	7.46	2.43	12.3	12.3	0.0
— <i>cota</i> L.	Annual	15.78	9.09	16.0	6.5	-9.5
— <i>austriaca</i> Jacq.	Annual	9.63	34.11	13.0	7.0	-6.0
<i>Artemisia absinthium</i> L.	Perennial	7.28	45.45	11.5	9.5	-2.0
— <i>annua</i> L.	Annual	4.05	14.13	10.5	7.7	-2.8

DNA content was measured in Feulgen-stained nuclei by the two-wavelength method of cytophotometry¹⁴; *Allium cepa* nuclei have been used for the conversion into absolute values. The percentage of DNA within the heterochromatic portion of the genome was calculated from measurements, after the extinction values for euchromatin had been set to zero¹⁵. The expected cell cycle times were calculated from the diagram given by Evans *et al.*². The actual duration was established after pulse labelling, by the method of labelled mitoses curves¹⁶. Striking negative differences between the expected and the actual cycle times can be seen in species with high amounts of heterochromatin-DNA, especially in some annuals.

because there is a linear relationship between these two parameters². In some of the species studied, however, the measured cycle duration differed significantly from the expected value (Table 1). In all annuals tested the cell cycle time was shorter than in the related perennials, in spite of the greater DNA content in three of the annual species. These three species have a proportionately greater amount of DNA as heterochromatin than their perennial relatives.

My results indicate that the nuclear DNA content can increase by the addition of heterochromatin without lengthening of the cell cycle. The cycle may even become shorter.

The percentage of heterochromatin within a given genome might, therefore, represent a correcting factor in the control of the duration of the cell cycle, in addition to the nuclear DNA content (the basic determinant). Heterochromatin is rich in repetitive DNA⁹ and replicates faster than euchromatin¹⁰. Thus annual species can become able to grow and develop rapidly, either by decreasing the nuclear DNA content or by increasing the DNA content through an increase in the percentage of heterochromatin. An increase in DNA, when found in specialised and progressive groups, is possibly always accompanied by a proportionally greater increase in repetitive sequences. Fox¹¹ reported, for example, that the heterochromatic portion of the genome in several beetles increased faster than did the euchromatic portion; increasing amounts of repetitive DNA were also found to be correlated with increasing DNA content in conifers¹². In *Vicia*, differences between species-specific DNA contents were reported to be caused by different numbers of rRNA genes, which appear in the form of heterochromatic chromosome segments¹³. Thus, the amount of heterochromatin is evidently an important factor in determining both nuclear DNA content and growth rate parameters. Details will be published in a forthcoming paper.

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Mode of action of soybean trypsin inhibitor (Kunitz) as a model for specific protein-protein interactions

THE protein trypsin inhibitors are proteins which bind very strongly to trypsin, blocking its active site ($K_i = 10^{-9}$ to 10^{-14} M). We have carried out a crystal structure analysis of the complex of soybean trypsin inhibitor (Kunitz) (STI), one of the largest inhibitors, with porcine trypsin. Huber and his colleagues have determined the structure of a complex of a small inhibitor, bovine pancreatic trypsin inhibitor (Kunitz) (PTI), with bovine trypsin¹. These studies improve our understanding of the catalytic mechanism of trypsin, demonstrate that various trypsin inhibitors act in a similar way and provide insight into the development of strong, specific binding between protein molecules.

From preliminary results on the crystal structure of isolated PTI², using available knowledge about chymotrypsin³ and trypsin⁴, it was possible to predict a unique mode of binding in which the inhibitor is oriented similarly to a true trypsin substrate^{4,5}. This prediction was confirmed by the crystal structure analysis of the complex of PTI with trypsin¹.

Native PTI is not detectably modified by trypsin, but cleavage of a specific peptide bond of STI (Arg 63'-Ile 64') is catalysed by trypsin. An equilibrium between STI and a modified form, in which this bond is hydrolysed, has been demonstrated⁶. This provides strong evidence that binding of STI to the catalytic site of trypsin mimics the productive mode of substrate binding.

The STI:porcine trypsin complex was crystallised and the first isomorphous derivative was discovered by H. T. Wright in this laboratory. The structure analysis has been taken to a resolution of 2.6 Å. The STI molecule, omitting certain uninterpretable regions, is shown in Fig. 1. As in the case of PTI, we observe that only a very small part of the molecule

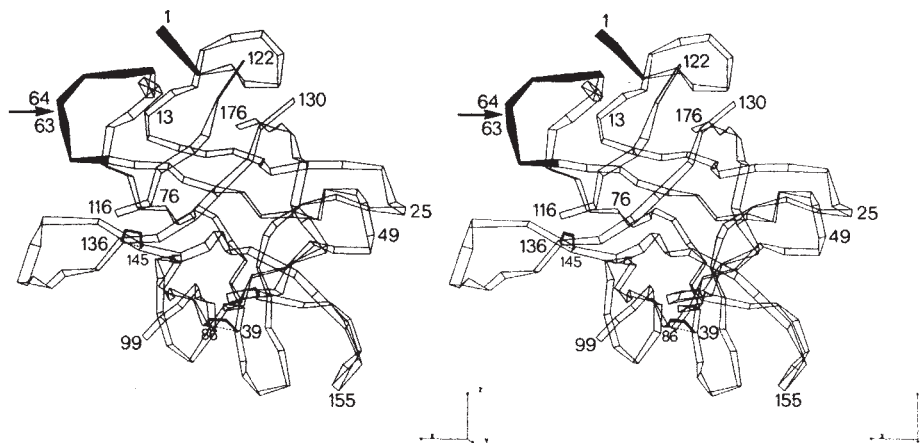


FIG. 1 Conformation of the polypeptide chain of soybean trypsin inhibitor as determined from the crystal structure of the complex with trypsin, and the amino acid sequence⁷. Residues 107'-115', 123'-129', 177'-181' could not be located in the electron density map; as a consequence, the identification of residues 116'-122' is tentative. The two disulphide

bridges are indicated. Residues which make contact with trypsin are blackened: the arrow indicates the scissile bond 63'-64'.

[Note added in proof. More accurate co-ordinates show that residues 13', 60', 71', 72' also make a few contacts with trypsin.]

is involved in the contact region. If the inhibition of trypsin is the function of these molecules, most of the protein does nothing more than stabilise the structure.

In the PTI complex a disulphide bridge adjacent to the active site effectively excludes access of water to the bond (Lys 15'-Arg 16') which lies at the active site of trypsin⁵. In the STI complex water is also excluded from the catalytic site, mainly by the chain (11'-14'), but this is held in place only by a few hydrogen bonds and van der Waals' contacts. The looser attachment may account for the measurable rate of hydrolysis of the STI complex of trypsin.

Both PTI and STI have a positively charged amino acid at P_1 to occupy the primary specificity pocket of the enzyme (Arg 63' in STI). The main chain NH of this amino acid is hydrogen-bonded to CO(214), as found in a chymotrypsin: substrate complex⁸. Interactions of adjacent residues in both the STI and PTI complexes (Fig. 2) agree with the interactions proposed for polypeptide substrates of chymotrypsin⁹⁻¹²: (a) from a study of models Segal *et al.*⁹ pointed out that a bulky group at P_2 can interact hydrophobically with Ile 99. This is observed in both PTI and in STI, where P_2 is Tyr 62'. (b) At P_3 which is Ser 61' in STI, the NH group is hydrogen bonded to CO(216) in both complexes. Segal *et al.*⁹ found a similar interaction for substrate analogues of γ -chymotrypsin. A second hydrogen bond which they found, involving NH(216), cannot occur in trypsin because it is differently oriented, as a consequence of the deletion of residue 218 (ref. 13). (c) In both complexes a residue in P_1' (Leu 64' in STI) makes hydrophobic contacts with the disulphide bridge 42-58. It was recently shown that interactions at P_1' accelerate deacylation of acylchymotrypsins by 50-100 times¹¹. There was no evidence of enhanced binding of the deacylating agent.

Structural models⁵ showed that the favourable interactions at P_1' could not occur in the Michaelis complex with a polypeptide substrate, because of unfavourable interactions of the substrate with the enzyme's active serine, Ser-195 (ref. 11). Similarly when the peptide bond P_1 - P_1' is hydrolysed, the residue at P_1' is forced away from P_1 by about 1 Å and the favourable interactions are again disrupted. In an intermediate state, when the carbonyl group of the scissile bond is bonded to both Ser-195 and its amide nitrogen, the substrate could make its best fit to the surface of the enzyme. This explained how the energy of this interaction could strain the enzyme:substrate complex toward the transition state, reducing the activation energy of acylation, rather than improving the binding constant¹¹.

In addition to the primary binding at P_1 , all three interac-

tions (a)-(c) are observed in both inhibitor complexes, which therefore both exhibit the mode of binding already inferred as the productive mode for trypsin substrates. The similarity of the interactions at P_3 - P_2 - P_1 - P_1' is confirmed by the close agreement of the recorded dihedral angles in the three intervening peptide bonds (Table 1), which must be close to experimental error. Although the agreement of chain conformation extends no further, one additional similarity exists: (d) In both PTI¹ and STI complexes, arginine is found at P_2' . In each case its guanidinium group lies parallel to Tyr-151 probably forming a charge-transfer complex. The guanidinium group is hydrogen bonded to CO-40. Despite this similarity, the main chain conformations at P_2' are

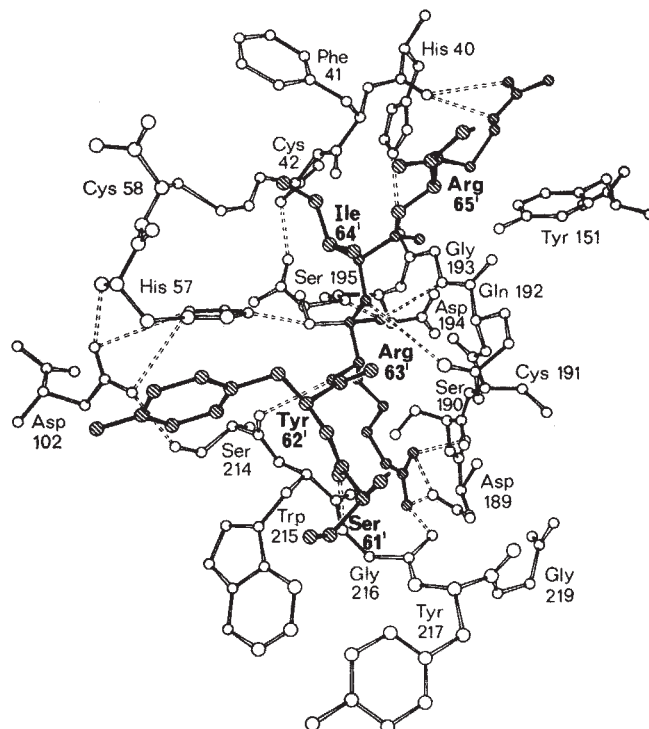


FIG. 2 Residues 61'-65' of soybean trypsin inhibitor and the parts of the trypsin molecule involved in binding them. Atoms of the inhibitor molecule are shaded and many of the interactions which they make are mentioned in the text. The carbonyl carbon of the scissile peptide bond 63'-64' is shown tetrahedrally coordinated. Trypsin residues Asn-97 and Leu-99 which lie in front of Asp-102 and touch the distal part of Tyr-62' have been omitted for clarity.

TABLE 1 Main chain conformations at the active site of bovine pancreatic trypsin inhibitor (Kunitz) and soybean trypsin (Kunitz)

Pancreatic	Trypsin	Inhibitor	Trypsin site	Soybean	Trypsin	Inhibitor
		Dihedral angles*				
				Dihedral angles		
Pro-13'	ϕ	- 86	P ₃	ϕ	- 46	Ser-61'
	ψ	- 28		ψ	- 21	
Cys-14'	ϕ	- 64	P ₂	ϕ	- 77	Tyr-62'
	ψ	152		ψ	136	
Lys-15'	ϕ	-116	P ₁	ϕ	- 89	Arg-63'
	ψ	87		ψ	85	
Ala-16'	ϕ	-135	P ₁ '	ϕ	-135	Ile-64'
	ψ	166		ψ	-115	
Arg-17'	ϕ	-116	P ₂ '	ϕ	-175	Arg-65'
	ψ	87		ψ	-179	

* R. Huber *et al.*, private communication

completely different in the two inhibitor complexes. Other trypsin inhibitors do not have arginine at P₂'.

Rühlmann *et al.*¹ were obliged to interpret the electron density at the active site of the PTI complex in the form of a tetrahedral intermediate, in which there is a covalent bond between the enzyme and inhibitor, whereas the peptide Lys 15'-Ala 16' at the active site is still linked. This tetrahedral form had been generally assumed to exist in the chemical pathway^{14,15} but in a normal hydrolysis reaction its lifetime is so short that it had never been convincingly demonstrated.

In Table 2 we give the distances observed in STI between C α (Arg 63'), C β (Ser 195) and C α (Ile 64'), together with the distances expected on stereochemical grounds for the various possible intermediate forms. The Michaelis and acyl forms both cause impossible crowding at the active site. We feel that this provides conclusive evidence that the STI complex is also in the tetrahedral form.

Structural data have indicated two aspects of the interaction between trypsin-like enzymes and their substrates which may favour the tetrahedral intermediate. One of these concerns the binding of the leaving group at P₁' and has been described above. The other concerns hydrogen bonds from the NH groups of Gly-193 and Ser-195 to the carbonyl group of the scissile bond, which are better oriented when the carbonyl carbon becomes tetrahedral¹⁶ and will be strengthened by any negative charge which exists on the oxygen. In the tetrahedral intermediate the buried negative charge, which may be thought of as originating from COO⁻ (Asp-102), is maximally delocalised across His-57 and the tetrahedral ester group.

The tetrahedral intermediate is close to the transition state

TABLE 2 Distances at the active site compared with expected distances for different intermediate forms of the complex

	C α (63')-C β (195) (Å)	C α (63')-C α (64') (Å)
Observed distance	3.7	3.9
Expected distances:		
Michaelis complex	5.2	3.8
Tetrahedral intermediate	3.7	3.8
Acyl enzyme	3.7	5.0

of the reaction^{17,18}. The essential requirement for a catalyst is to stabilise the transition state¹⁹ and an effective enzyme must provide a means of doing this²⁰. The two trypsin inhibitors do this so effectively that the tetrahedral intermediate constitutes a minimum of free energy in the reaction pathway, and at least in crystals it becomes the most abundant state. Formation of a covalent bond between the enzyme and the inhibitor does not in itself explain the strong binding, as is shown by the high affinity of PTI, STI and other inhibitors for anhydrotrypsin, in which the oxygen of Ser-195 has been removed^{21,22}. In anhydrotrypsin the overcrowding at the active site is relieved, and the intact inhibitor can bind favourably to the active site. Calorimetric measurements show that the binding of STI to trypsin is endothermic²³, reflecting the relatively high enthalpy of the tetrahedral form, but a large entropy term stabilises the complex.

The entropy of association between protein molecules derives largely from the shells of immobilised, or partly ordered, water molecules which surround the two separate molecules. These are reduced in volume on association²⁴. An external polar group is normally hydrogen-bonded to a water molecule, which becomes immobilised. On association, provided that such hydrogen bonds are exchanged for an intermolecular hydrogen bond, there is little change of enthalpy; but the release of immobilised water molecules may be an important contribution to the entropy of association.

There is an important difference between the association of an enzyme with a folded protein molecule which makes a perfect fit, and the binding of a loosely coiled molecule. When a small polypeptide substrate binds to the enzyme, its loss of internal degrees of freedom reduces the free energy of binding. Binding of a correctly folded protein molecule may be achieved without change of internal freedom. These entropic factors, even over the small interaction areas observed in PTI and STI, are evidently sufficient to yield the high association constants which are observed.

Enzyme-substrate interactions have evolved to lower the activation energy of a chemical reaction step. These studies show in detail how the energetics of these interactions may provide the driving force for very strong binding, with a highly specific recognition system. To evolve a receptor which recognises a specific effector and signals its presence to a linked biochemical system, one would require in addition that substrate binding triggered a structural change.

TABLE 1 Angiotensin II-induced changes in mean aortic blood pressure, uterine blood flow and concentrations of 'PGE' in uterine venous blood

Dog	Dose Ang. II* (ng kg ⁻¹ min ⁻¹)	Mean aortic blood pressure		Uterine blood flow†		'PGE'	
		Control (mm Hg)	Ang. II (mm Hg)	Control (ml min ⁻¹)	Ang. II (ml min ⁻¹)	Control (ng ml ⁻¹)	Ang. II (ng ml ⁻¹)
1	10	85	95	69	72	—	—
2	20	80	95	157	186	0.49	0.56
3	10	105	135	100	181	0.21	0.55
4	37.5	110	125	33	44	0.11	0.22
5	37.5	95	105	115	155	0.18	0.92
6	37.5	100	125	52	63	0.23	0.33
7	20	90	115	62	76	—	—
8	37.5	135	145	—	—	0.37	0.46
$\bar{x}$		100	118	84	111	0.26	0.51
s.e.		±6	±6	±16	±23	±0.06	±0.10

* Angiotensin (Ang.) II was infused intravenously.

† Represents blood flow to one horn of the uterus.

tained with arachidonic acid, the precursor of PGE₂¹⁶, which has also been shown to release PGE₂¹⁴. Thus, arachidonic acid increased UBF when given before, but not after indomethacin.

The levels of 'PGE' that we found in uterine venous blood are more than ten times those reported in the venous effluent of the hind limb⁶ and are equal to or greater than those in renal venous blood¹⁷. The capacity of the kidney to synthesise prostaglandins is surpassed only by the seminal vesicle¹⁸ and in view of our findings, perhaps by the gravid uterus as well. Since prostaglandins of the E series have been shown to oppose the vasoconstrictor activity of pressor systems⁸, our findings of high uterine venous levels of 'PGE' which are increased further by angiotensin II may account in part for the increased UBF produced by angiotensin II and the altered reactivity of the uterine vasculature to other vasoconstrictor stimuli. Renin, which is found in abundance in the pregnant uterus¹⁹ has been suggested to participate in the control of uterine vascular resistance³, possibly through the generation of an angiotensin which in turn stimulates uterine prostaglandin synthesis. In this way, angiotensin-prostaglandin interactions may participate in the regulation of uterine blood flow. The importance for the uterine circulation of uninterrupted synthesis of prostaglandins by the gravid uterus was revealed after inhibition of prostaglandin synthetase with indomethacin²⁰. Uterine blood flow fell to levels less than 30% of control. Simultaneously, the concentration of "PGE" in uterine venous effluent declined to a mean value less than 10% of control.

This work recalls our previous studies on the renal circulation: (1) continued production of PGE₂ was shown to maintain resting renal blood flow⁶, and (2) angiotensin II and noradrenaline were shown to increase renal synthesis of PGE₂ above basal levels⁸. The importance of this function of the kidney was revealed after inhibition of prostaglandin synthetase by indomethacin. Thus, resting renal blood flow decreased by more than 40%⁶ and the renal vasoconstrictor action of angiotensin II and noradrenaline increased several-fold²¹. The previously reported ameliorating effects of pregnancy on both experimental hypertension²² and essential hypertension²³ may reside partially in the capacity of the gravid uterus to synthesise one or more substances which have antihypertensive properties, such as PGE₂²⁴. Finally, we have not distinguished the site of origin within the uteroplacental circulation of the 'PGE' which appears in uterine venous blood. One or more sites including the foetal as well as the maternal components of the placenta, nor excluding the myometrium, should be considered. Thus, we were not able to relate changes in uterine blood flow after indomethacin to a primary effect on one or more of the several elements of the uteroplacental circulation.

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Role of *rel* gene in translation during amino acid starvation in *Escherichia coli*

It has been thought that the *rel* gene in *Escherichia coli* is responsible for the stringent control of stable RNA synthesis dependent on a supply of amino acids, since the gene was identified as the site of mutation to the relaxed phenotype¹. But in our previous reports^{2,3}, we suggested that mutational defects in *rel*⁻ cells are probably in the translational machinery, the integrity of which may be required for stringent control. This supposition is based on the following observations: (1) When *rel*⁻ cells are shifted down in carbon or nitrogen source, the stringent control of RNA synthesis is observed in spite of their *rel*⁻ genotype^{2,4}. Only when deprived of amino acids do *rel*⁻ cells fail to restrict RNA synthesis, in contrast to cells having the *rel*⁺ allele. (2) Ribosomal inhibitors like chloramphenicol abolish the stringent control in both *rel*⁺ and *rel*⁻ cells^{2,5,6}. Furthermore, a cold-sensitive mutant defective in protein synthesis at low temperature carrying a mutation in *spcA* locus, also shows the relaxed phenotype under non-permissive conditions⁸. It has been reported that several other mutants of *rel*⁺ strains having temperature-sensitive phenylalanyl-tRNA synthetase⁷, peptidyl-tRNA hydrolase⁸, or elongation factor G (ref. 9) all failed to control the synthesis of RNA at the higher temperature. This suggests that normal synthesis of protein is re-

quired for stringent control. (3) During shift-down of a carbon or nitrogen source there is no difference between the growth of *rel*⁺ and *rel*⁻ cells. Only after shift-down of amino acids do *rel*⁻ cells require a much longer lag period before they resume growth^{2,10}. This is due to the inability of *rel*⁻ cells to carry out the normal synthesis of protein when the supply of amino-acids is limited.

The work described here was carried out to extend our previous reports^{2,3} that the *rel*⁻ mutant is defective in translation under amino acid deprivation. We found that polyosomes in the amino acid-starved *rel*⁺ cells function actively in protein synthesis while in *rel*⁻ cells they are inert.

When the cells growing in an amino acid-rich medium are shifted down to one poor in amino acids, *rel*⁺ cells are able to resume growth much more rapidly than *rel*⁻ cells². Enzymes responsible for the synthesis of amino acids are produced immediately after shift-down in *rel*⁺ cells, but not in *rel*⁻ cells². Formation of several other enzymes including β -galactosidase¹¹, alkaline phosphatase¹², and ornithine transcarbamylase¹³ was also impaired in *rel*⁻ cells during amino acid starvation.

When the cells were shifted down to an amino acid-poor medium, the overall rate of protein synthesis was reduced to less than 10% of that in pre-shift conditions in both *rel*⁺ and *rel*⁻ strains (see zero time in Fig. 1a and b). But as shown in Fig. 1a the rate of protein synthesis in *rel*⁺ cells recovered rapidly during incubation in minimal medium and reached a plateau which was sufficient to maintain the growth of cells in shift-down conditions. On the other hand, the rate of protein synthesis in *rel*⁻ cells gradually declined (Fig. 1b). The potential protein synthetic activity as measured by incorporation of ¹⁴C-phenylalanine in the presence of a mixture of twenty amino acids was preserved in *rel*⁺ cells during amino acid starvation, while in *rel*⁻ cells it decreased.

The change of the rate of RNA synthesis following the amino acid shift-down is also shown in Fig. 1. After deprivation of amino acids, RNA synthesis in *rel*⁺ cells was extremely

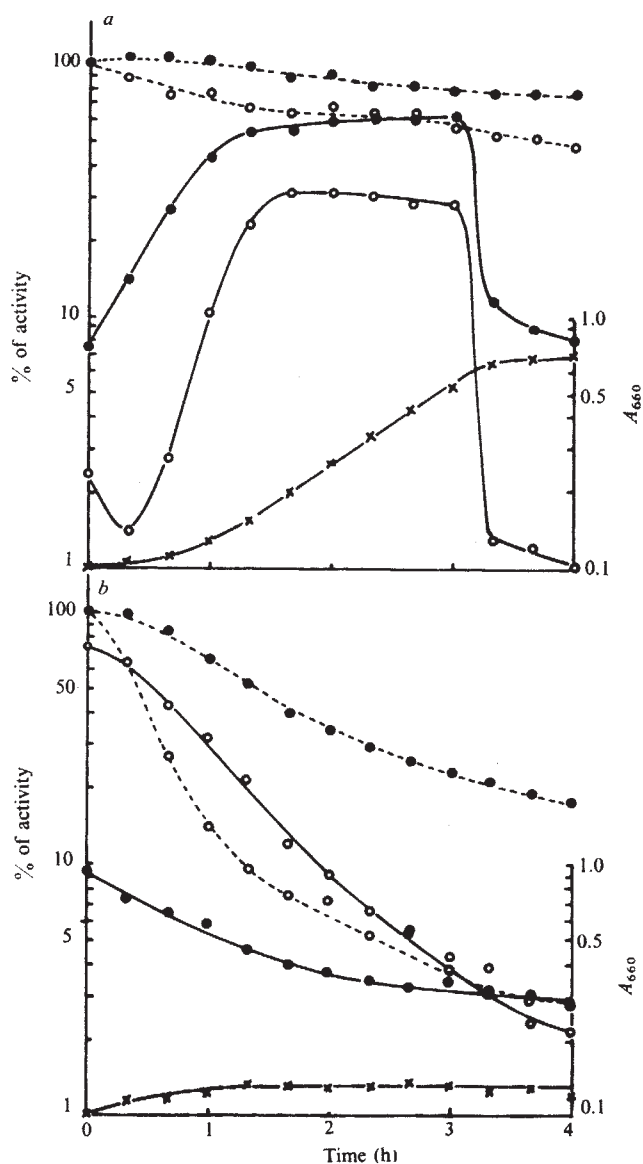


FIG. 1 Change in protein and RNA synthesis in *rel*⁺ and *rel*⁻ cells following the shift-down of amino acids. CP78(*rel*⁺) and CP79(*rel*⁻), isogenic except for the *rel* locus¹⁴, were used. They are descendants of K12 strain and require leucine, threonine, histidine, arginine, and thiamine. Cells were grown at 37°C with shaking in glucose (0.4%) required amino acids (20 μ g ml⁻¹ each), thiamine (2 μ g ml⁻¹), M9 medium; further supplemented with all twenty L-amino acids (0.1 mM each). The cultures grown to log phase were centrifuged, washed and resuspended in a fresh glucose-M9 medium containing only required amino acids and thiamine. During incubation in the shift-down medium, at the time indicated aliquots were withdrawn and the activities of protein and RNA syntheses, and the turbidity at 660 nm (1 cm light path) were estimated. For the measurement of protein synthesis, two samples (0.5 ml each) were taken and pipetted into two different test tubes; one contained 15 μ l of ¹⁴C-phenylalanine (5 μ Ci ml⁻¹; 0.25 μ mol ml⁻¹) and 50 μ l of distilled water, and the other contained 15 μ l of ¹⁴C-phenylalanine and 50 μ l of nineteen L-amino acids mixture except phenylalanine (1 mM each). For RNA synthesis, 0.5 ml samples were added to the test tubes containing 15 μ l of ³H-uracil (5 μ Ci ml⁻¹; 0.25 μ mol ml⁻¹) with or without 50 μ l of a mixture of twenty amino acids (1 mM each) as above. After incubation for 10 min at 37°C, 3 ml of ice-cold 5% trichloroacetic acid was added. The precipitates were collected by filtration on Whatman GF83 glass fibre papers, washed three times with 3 ml of 5% trichloroacetic acid and then once with acetone. Radioactivity was determined in a liquid scintillation spectrometer using toluene-based counting mixture. Activities of protein and RNA syntheses were represented by the radioactivities incorporated per A₆₆₀ nm of the culture. Solid lines indicate the activities which were measured by the incorporation of ¹⁴C-phenylalanine or ³H-uracil without addition of amino acids mixture. Dashed lines show the activities measured with addition of amino acids mixture (potential activity). a, CP78 (*rel*⁺); b, CP79 (*rel*⁻). ●, protein synthesis; ○, RNA synthesis; ×, cell growth.

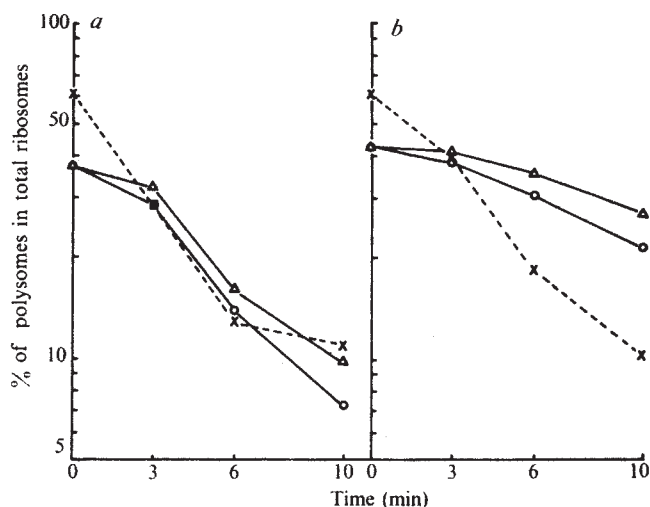


Fig. 2 Decay of polysomes in CP78 (*rel*⁺) and CP79 (*rel*⁻) in the presence of rifampicin. Cells, grown to log phase in a glucose-M9 medium supplemented with twenty amino acids, were collected, washed and resuspended in M9-buffer to approximately 7×10^8 cells ml. The cell suspension was poured into the flasks containing 0.6 ml of glucose (0.2 g ml⁻¹), 0.6 ml of thiamine (10 μ g ml⁻¹), 0.6 ml of mixture of required amino acids (0.1 mg ml⁻¹ each), and 3 ml of mixture of twenty amino acids (1 mM each) when specified. The total volume was adjusted to 30 ml. After incubation for 10 or 30 min 1 ml of rifampicin solution (6 mg ml⁻¹) was added to each flask. The incubation was stopped by pouring over crushed ice at indicated times. Cell lysates were prepared according to the method of Godson¹⁷. The cells were collected by centrifugation and resuspended in 0.4 ml of 25% (w/v) sucrose solution containing 10 mM Tris-HCl (pH 8.1). Then to the cell suspension were added 50 μ l of lysozyme (1 mg ml⁻¹) and 50 μ l of EDTA (10 mM). After mixing by gentle swirling for 3 min in the cold, 0.5 ml of lytic mixture was added, which contained 40 mM Tris-HCl (pH 8.1), 1% (w/v) Brij-58, 20 mM MgCl₂, 0.2% (w/v) deoxycholate and 1 μ g ml⁻¹ DNase. Lysed solutions were centrifuged for 10 min at 10,000 r.p.m. to remove the cell membranes and debris. The lysates (3.4 A₂₆₀ unit) were analysed in 4.6 ml of 0.4 M to 1.3 M linear sucrose density gradients containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 60 mM KCl. Gradients were centrifuged in Spinco SW 50.1 rotor for 50 min at 45,000 r.p.m., then unloaded from the top with ISCO gradient fractionator, which monitored the optical density at 254 nm. The polysome content was determined by cutting and weighing the tracings after correction of the base lines. a, CP78 (*rel*⁺); b, CP79 (*rel*⁻). X, Cells were incubated with twenty amino acids for 10 min before addition of rifampicin; O, incubated in the medium containing only required amino acids for 10 min; Δ, incubated in the medium containing only required amino acids for 30 min.

suppressed, but it started to rise rapidly before the recovery of growth (Fig. 1a). The initial suppression of RNA synthesis in *rel*⁺ cells is the consequence of stringent control. In the case of *rel*⁻ cells, the rate of RNA synthesis was not restricted by deprivation of amino acids but during incubation in the minimal medium it declined gradually (Fig. 1b). Potential RNA synthesis, like protein synthesis was preserved in *rel*⁺ cells, but *rel*⁻ cells were not able to maintain the activity at pre-shift level.

We have suggested that the regulation of RNA synthesis observed in *rel*⁺ bacteria is dependent on the function of protein synthesising machinery under shift-down conditions^{2,3}. It seems that the cells having the *rel*⁺ allele have the ability to carry out protein synthesis under a limiting supply of amino acids. This teleological significance of the function of the *rel* gene was not considered until the shift-down experiment was carried out, since in the experiments in which required amino acids are withheld, the amino acid auxotrophs are not able to grow (see refs 2 and 15).

When proteins are being synthesised actively in the cells,

polysomes are in a dynamic state, being rapidly formed and degraded. Rapid degradation of the active polysomes can be observed when the synthesis of messenger RNA is blocked by addition of actinomycin or rifampicin. On the other hand, polysomes are stabilised and accumulated when ribosome movement on messenger RNA is arrested by ribosomal inhibitors such as chloramphenicol or fusidic acid (see ref. 16). The translational activity on polysomes can therefore be estimated by measuring the stability of polysomes after inhibition of messenger RNA synthesis by rifampicin.

We have measured the stability of polysomes of both *rel*⁺ and *rel*⁻ cells in either growth or amino acid shift-down conditions in the presence of rifampicin (Fig. 2). Polysomes in the actively growing cells of both *rel*⁺ and *rel*⁻ strains are degraded rapidly with halflife of about 3 to 4 min but the stability of polysomes in amino acid-deprived cells is distinctly different in *rel*⁺ and *rel*⁻ strains. Polysomes in amino acid-deprived *rel*⁺ cells decay almost at the same rate as these in the growing cells although there is a lag before degradation starts (Fig. 2a). In a *rel*⁻ strain, polysomes of cells incubated in an amino acid-deprived medium are much more stable than those of growing cells (Fig. 2b). The stability of polysomes is increased by prolonged incubation in the minimal medium.

These findings clearly indicate that the translation on polysomes in *rel*⁺ cells is occurring actively even under the limiting supply of amino acids, whereas in amino acid-deprived *rel*⁻ cells polysomes are inert, as when the ribosome movement is arrested by addition of chloramphenicol.

Ron¹⁸ and Silengo¹⁹ have reported essentially the same results as ours on polysome turnover in *rel*⁺ and *rel*⁻ bacteria. But Cozzzone and Donini²⁰ have reported a conflicting result: that when *rel*⁺ and *rel*⁻ cells carrying temperature-sensitive valyl-tRNA synthetase are treated with rifampicin at a higher temperature, polysomes in both *rel*⁺ and *rel*⁻ cells decay as rapidly as those in growing cells under permissive conditions.

From the results of polysome turnover, it was expected that, under the amino acid-starved condition, proteins are synthesised normally on polysomes and released to the soluble fraction in *rel*⁺ strains, but not in *rel*⁻ strains. To prove this, we have carried out the following chase experiment. The amino acid-starved cells of both *rel*⁺ and *rel*⁻ strains were labelled for 1 min with ³H-amino acids of high specific activity. After labelling, an excess of unlabelled amino-acids was added. Figure 3A shows the kinetics of incorporation of radioactive amino acids into the cell lysate and its acid-insoluble fraction with *rel*⁺ and *rel*⁻ cells. Although the specific activities of amino acids were reduced about a thousand-fold by addition of unlabelled amino-acids, the incorporation of radioactive amino acids into the acid-insoluble fraction was not suppressed immediately. In this experiment the amino acid pool of the cells contained radioactivity more than ten times higher than the protein fraction, since the protein synthesis was greatly reduced by amino acid starvation. Therefore, even after the addition of excess unlabelled amino acids there is a time lag before the labelled nascent polypeptides on polysomes are replaced by the unlabelled polypeptides (see Fig. 3C).

Figure 3B shows the pattern of sucrose density gradient centrifugation of lysates of *rel*⁺ and *rel*⁻ cells which were chased for 30 and 90 s by the addition of an excess of unlabelled amino-acids. Fig. 3C shows the time course of the incorporation of radioactive amino acids into the acid-insoluble fraction of polysomes, ribosomes, and supernatants calculated from the results of Fig. 3B. As expected, after the addition of unlabelled amino acids the labelled nascent polypeptides on polysomes in amino acid-starved *rel*⁺ cells were displaced by unlabelled polypeptides and released to the supernatant fraction, while in amino acid-starved *rel*⁻ cells the radioactive polypeptides were retained on polysomes.

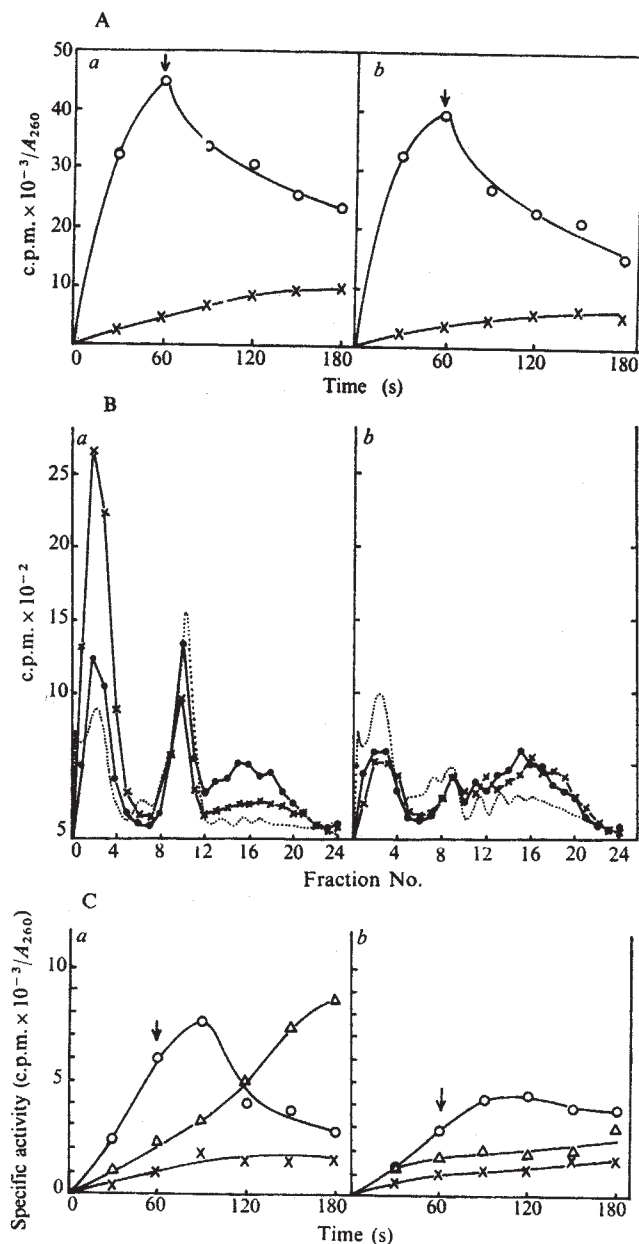


FIG. 3 Pulse labelling of proteins in amino acid-starved cells of both CP78 (*rel*⁺) and CP79 (*rel*⁻). A 60 ml culture, grown to log phase under the supply of twenty amino acids, was washed and resuspended in a glucose (0.4%)-M9 minimal medium without addition of any amino acid. After starvation for 15 min at 28°C, 10 μ Ci each of radioactive amino acids mixture (8.7 Ci mmol⁻¹ ³H-L-phenylalanine (G), 28.7 Ci mmol⁻¹ ³H-L-lysine-4,5 and 42 Ci mmol⁻¹ ³H-L-tyrosine-3,5 was added. After 60 s of labelling, unlabelled phenylalanine, lysine and tyrosine were added to the final concentration of 25 μ M each. At 30 s intervals 10 ml portions of the culture were poured over ice containing 1 mg of chloramphenicol. The cell lysates were prepared and analysed by sucrose density gradients as in Fig. 2. For the determination of radio-activity, 0.2 ml fractions of the gradient were collected and precipitated with 2.5 ml of cold 4% perchloric acid. They were then filtered on Whatman GF83 glass filter papers, washed with 0.4% perchloric acid, 50% ethanol and dried. Radioactivity was determined by scintillation counting in a toluene-based mixture. a, CP78 (*rel*⁺); b, CP79 (*rel*⁻). A, Incorporation of radioactive amino-acids into cell lysate and its acid-insoluble fraction. The arrow indicates the time when unlabelled amino-acids were added. ○, Lysates were applied directly to glass filter papers, dried and counted; ×, lysates were precipitated with cold 4% perchloric acid and counted as described above. B, Sucrose density gradient pattern of incorporation. Dotted line shows optical density. ●, The radioactivities were chased for 30 s by addition of unlabelled amino acids after labelling with ³H-amino-acids for 60 s; ×, chased for 90 s. C, Release of radioactive polypeptides from polysomes to supernatant fraction. Specific activities of each fraction of polysome, ribosome and supernatant were calculated from the patterns of sucrose density gradients, which denote total counts of corresponding region per total A_{260} unit of that region. The arrow indicates the time when unlabelled amino acids were added. ○, Radioactivities in polysome region; ×, ribosome region; Δ, supernatant region.

lend additional support to the view that *rel* gene product functions at the translational level during amino acid starvation. Hall and Gallant²² have also found that an abnormal β -galactosidase with reduced specific enzymic activity and increased thermolability is formed in amino acid-starved *rel* cells.

Pedersen *et al.*²³ and Haseltine and Block²⁴ have reported that ppGpp and pppGpp, the compounds which accumulate in response to amino acid starvation in *rel*⁺ cells, are synthesised on ribosomes in a cell-free system of *E. coli*. The synthesis requires the presence of messenger RNA, deacylated tRNA, and a protein factor isolated from the ribosomal washing of *rel*⁺ cells²⁵. Their results suggest that ppGpp and pppGpp are formed on polysomes, when deacylated tRNA is bound to the A site of ribosomes due to the unavailability of the appropriate aminoacyl-tRNA species.

As is shown here, polysomes in *rel*⁺ cells are able to continue to synthesise proteins after amino acid deprivation, whereas in *rel*⁻ cells the movement of polysomes is inhibited. It would be of interest to examine whether there is any close correlation between the formation of the unusual guanine nucleotides and the successful use of limiting amount of aminoacyl-tRNA during deprivation of amino acids.

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The above results are consistent with those obtained from polysome turnover experiments. Thus, the translational defect in *rel*⁻ cells under the restriction of amino acid supply may be located in some processes involving ribosomal functions.

Many studies have been reported on the mechanism of induction or derepression of enzymes in bacteria. In most cases, the induction or derepression of enzymes is achieved by the regulation at the level of transcription, and the mechanism involved in this process is largely understood. The mechanism of translational control in enzyme formation during adaptation has not yet however been clarified.

From the study on the formation of β -galactosidase in amino acid-starved cells of *Salmonella typhimurium*, Engbæk *et al.*²¹ have pointed out that a mechanism for successful use of a limiting supply of aminoacyl-tRNA must exist in the cells. We consider that the *rel* gene is responsible for this mechanism. As we have shown before², the mutation in the *rel* gene causes the impairment of formation of enzymes when the supply of amino acid is limited. We have observed that polysome turnover is suppressed in *rel*⁻ cells under amino acid starvation, whereas in *rel*⁺ cells polysomes actively turn over and the polypeptides synthesised on polysomes are transferred normally to the supernatant fraction. These results

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Selective pressure on HL-A polymorphism

THE advantages of heterozygotes is one of the most frequent explanations of the maintenance of a polymorphism. This advantage may have the form of a lower fitness of all homozygotes, whatever the allele they possess. According to observations on a small Tuareg isolate, such is apparently the case for the polymorphism of the histocompatibility system HL-A.

The HL-A system (human leukocyte antigen A) is immunologically detected by means of specific sera. It depends on two loci which lie close together on the same chromosome. At the present time (including the blanks), 15 alleles are well defined at the first locus (SD1) and 20 alleles at the second (SD2). Each combination of one allele at the first locus and one at the second is called a 'haplotype'. The number of these combinations is therefore 300. Because of the low recombination fraction between the loci (about 0.008) (ref. 1), the system can, for certain purposes, be considered as a single locus system with 300 alleles.

Homozygotes (having two identical haplotypes) are very rare since all the haplotypes have a lower frequency than 10% in Caucasoid populations. It is pointless to try to show a discrepancy between the observed frequency of homozygotes and that expected in a Hardy-Weinberg equilibrium. The only exception is in very small, isolated populations in which, because of founder effect or genetic drift, the number of haplotypes present in the genetic makeup of the group is greatly reduced.

This is the case with the Kel Kummer Tuareg tribe, of the South Sahara. This tribe was founded in the seventeenth century, since when it has lived entirely apart from surrounding populations and has rigorously respected the very strict rules of mating which require marriage with the mother's brother's daughter. At present it numbers about 300 people.

The genealogies, collected by A. Chaventré² give an exhaustive description of the descent of the tribe, and some of the process of transmission of the genetic heritage through three centuries. They cover a total of 2,500 individuals, and an example of such a genealogy is shown in Fig. 1.

These genealogies gathered from oral tradition, have been confirmed by many cross checks with written records (military registers and accounts of travellers), systematic research on paternity exclusion and research on common ancestors of individuals with rare genes. These verifications have shown the genealogical information to be reliable. The 2,500 individuals, dead or living, of the tribe all descend from a few ancestors, with extremely intricate lines of descent.

This inbreeding may be measured by the classical F coefficient³. It is possible to calculate the F values by computer taking into account all the possible paths between living individuals and their common ancestors (in some cases the number of paths is about 10^6). For the set of 106 individuals whose HL-A genotype is known, the mean of the computed F coefficients is 0.1108, which is extremely high. In two sibships it is even greater than 0.24 ($F = 0.25$ for offspring of sib-mating).

The homogeneity that results from this inbreeding is better shown by taking the 'probabilities of a gene's origin', that is, the set of probabilities that, for the various founders, a gene taken at random in the population is derived from him, where a 'founder' is an ancestor whose parents are unknown³.

Classifying the 97 founders in order of decreasing importance, we obtain Table 1. This shows that 90% of the genetic makeup of the tribe comes from only 22 individuals. These individuals represent 44 haploid sets, some of which are probably identical since the founders certainly were related (but in a way we do not know). It is therefore evident that the Kel Kummer Tribe exhibits an uncommon genetic homogeneity.

To discover the HL-A genetic structure of the Kel Kummer tribe we tested for antigens: HLA1, 2, 3, 9, 10, 11, W29, W30+31 and W28 for the first locus (SD1), and HLA5, 7, 8, 12, 13, W5, W10, W14, W15, W17, W18, W21, W22, W27 and DA(6) for the second locus (SD2). The research^{4,5} on the HL-A antigens of the Kel Kummer have shown that they possess only 21 haplotypes, two of which exist at high frequencies, namely: HL-A11/W21, 19%; W28/HL-A7, 17%.

Among the 106 individuals whose genotypes (the two haplotypes) are known, only one is a possible homozygote; but this cannot be ascertained since he may have a blank allele at both loci; a study of his genealogy gives the probability 0.50 of homozygosity.

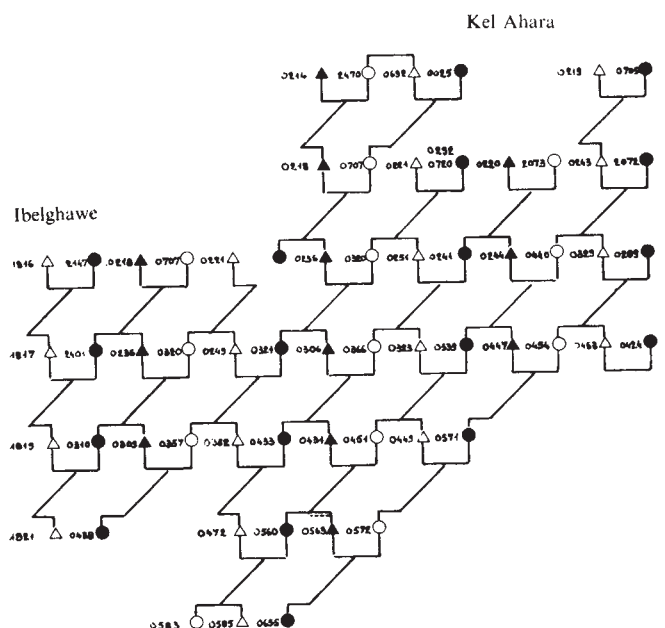


Fig. 1 Extract of Kel Kummer genealogy.

TABLE 1 Genetic make-up of the Kel Kummer tribe

Founder No.	Proportion of the genetic make-up (%)	Cumulative
1	10.6	
2-7	41.8	52.4
8-12	19.4	71.8
13-22	17.4	89.3
23-97	10.7	100

The number of homozygotes we expected is, however, significantly higher. If the population were in Hardy-Weinberg equilibrium for the HL-A loci, the total frequency of homozygotes, equal to the sum $\sum p_i^2$ of the haplotypes' squared frequencies, would be 8.4%. Moreover we have seen that the rules of choice of mate are very strict and so the prohibition of sib-mating, which entails a small 'negative inbreeding', is largely counterbalanced by the preference for cousin mating (30% of the marriages) or for mating with a cousin once removed (15%). The mating behaviour for the whole tribe corresponds to a deviation from random mating measured by the δ coefficient³, such that $\delta = +0.01$. The frequency of homozygotes, knowing the present genetic structure and the mating behaviour should therefore be

$$h = \delta + (1 - \delta) \sum p_i^2 = 9.3\%$$

Under the hypothesis that the observed sample is a random sample, the expected number of homozygotes among the 106 tested individuals, is a random variable with expected value $E = 106 \times 0.093 = 9.86$ and standard deviation $\sigma = (106 \times 0.093 \times 0.907) = 2.99$. The observed number (1 or maybe 0) is thus largely outside the confidence interval ($P < 0.005$).

The mean inbreeding coefficient of the tested individuals is $F = 0.1108$. Each of them has therefore a probability of greater than 0.11 of being a homozygote. Because of the non-independence of the genes of the various individuals, one cannot consider the number of individuals with two identical genes as a binomial variable. There is an inconsistency between the expected number of such individuals ($0.1108 \times 106 = 11.7$) and the observed number of homozygotes.

This observation supports the hypothesis that the HL-A chromosome segment is maintained in a polymorphic condition by disadvantages to being a homozygote, whatever genes are possessed. A model of this kind has been proposed for the histocompatibility systems⁶, but has not previously been supported by observation. The present data do not enable the role of HL-A system itself to be distinguished from that of a linked locus. It is probable however that HL-A antigens are present, in the haploid state, on the spermatozoa⁷: however, no disturbance on zygotic assortment for the HL-A system has been found in family studies^{8,9}.

Finally we note that the genes of the main system of histocompatibility, HL-A, are located on a chromosome segment which is very important for many immunological processes (locus of mixed lymphocyte culture, MLC, and probably locus of immune response, Ir). The detection of the presence of foreign cells (alloimmunisation) depends mainly of this segment; it is possible that it includes 'archaic' loci, which have remained unchanged during evolution, together with duplicated loci which have become more and more sophisticated. In plants there exist loci for detection of foreign cells, the 'self-sterility systems', which prohibit homozygous fertilisation and maintain polymorphism. It is possible that such genes still exist in man on the alloimmunisation chromosome segment, contributing to the maintenance of polymorphism. This interpretation of our observations is supported by the following argument: when non-related parents share the same allele for HL-A or for MLC loci, the offspring is homozygote with a frequency

close to the expected proportion¹⁰; consequently the 'prohibition of homozygote' we have observed may correspond to a locus closely linked to HL-A but which is not HL-A itself nor MLC.

This homozygous deficit has not, however been found in studies of other relatively isolated populations¹¹. This leads us to believe that the gene preventing homozygosity (which is not HL-A) may be at some distance from the HL-A loci on this chromosome. Nevertheless in the Tuareg tribe, which is extremely inbred, it is always transmitted with the HL-A loci because of the limited recombination opportunities. Another theory would be that the locus linked to HL-A possesses several alleles, not one of which allows homozygosity. This has been proven to be the case of T locus in mice which is linked to the major histocompatibility locus (H-2).

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Stimulation of bone marrow haemopoietic stem cells by a factor from activated T cells

I present here direct evidence that a factor(s) produced by activated thymus-derived lymphocytes (T cells) enhances proliferation of pluripotent bone marrow stem cells.

T cells have been known as a source of humoral mediators which can regulate (enhance or inhibit) antibody response¹⁻⁹. These mediators are released when T cells are stimulated with an antigen⁶⁻⁹ or with mitogens (phytohaemagglutinin (PHA) or concanavalin A) although direct evidence for the latter is not available¹⁰. With one exception⁹, the effect of the mediator was reported to be non-specific, that is, factor that was induced by one antigen stimulated production of antibody to another non-cross-reacting antigen⁶⁻⁸. This implies that the factor stimulates proliferation and differentiation of immunocompetent bone-marrow-derived cells (B cells) in general.

TABLE 1 Activation of splenic haemopoietic c.f.u. by PHA *in vitro* and *in vivo*

Source of spleen cells	PHA*	Recipient mice No. cells† injected, × 10 ⁶	c.f.u. per spleen‡, individual mice	c.f.u. per 10 ⁶ injected cells (fresh or cultured)	³ H-thymidine pulse§ (d.p.m. per 10 ⁶ cells)
Fresh suspension					
normal donors	—	2.0	3, 3, 4, 4, 5	19.0 (15–25)¶	
PHA-injected donors	+	2.0	9, 12, 13, 14, 15	63.0 (45–75)	
Cell culture	—	15.0	0, 0, 0, 0, 1, 2, 2, 2, 3	≤1.0 (<1–2)	1,950 (1,900–2,000)Ⓢ
	+	10.0	2, 2, 3, 3, 3, 3, 4, 7	3.4 (2–7)	117,500 (94,300–144,000)

Male Balb/c mice (Jackson Laboratory) were both donors (6–8 weeks old) and recipients (10–12 weeks old). Recipients received an X-ray dose of 700 rad (180 kV, 15 mA, 80 rad min⁻¹, with 2 mm Al and half-value layer 0.5 mm Cu, distance 51 cm) 6 h before cell transfer. A cell suspension (pooled from several donors) was prepared by pressing spleens through stainless steel wire screens in cold Puck's saline with antibiotics, followed by washing. Eagle's minimal essential medium was used for suspension cultures, containing 1% non-essential amino acids (100x), 2mM L-glutamine, 1mM sodium pyruvate, 1% antibiotic mixture (100x) and 5% foetal calf serum. Cultures (2–3 ml, 10⁷ cells per ml), in disposable tissue culture tubes, (Falcon No. 2051) were kept for 3 d at 37°C in air with 5% CO₂. Cells were then washed and injected into irradiated recipients in the numbers indicated. PHA was added at a concentration of 2.5 µg ml⁻¹ of medium, at the beginning of cultivation. For *in vivo* experiments, donor mice were injected with 200 µg intraperitoneally 24 h before cell transfer.

† Nucleated cells (viability determined by trypan blue exclusion).

‡ c.f.u. is the number of macroscopic colonies visible on spleen surface 8 d after cell transfer. Colonies were counted using magnifying glasses (10x) after brief immersion of the spleen into acetic acid-ethanol (1:4). No colonies were found in spleens of control irradiated recipients which receive no cell inoculum.

§ Pulse labelling was done in 2 ml cultures containing 10⁶ cells per ml of the medium with or without PHA (0.5 µg ml⁻¹). ³H-thymidine (2.5 µCi ml⁻¹ of culture) was added for 24 h between the second and third days of cultivation. Cells were then precipitated and washed with 0.2 N perchloric acid, the pellet resuspended in 0.1 N NaOH, aliquots (0.1 ml) pipetted into scintillation vials with 10 ml of Aquasol (New England Nuclear Corp.) and radioactivity determined in a Packard scintillation counter.

¶ Average number of c.f.u. per 10⁶ of injected cells (range within the group).

Ⓢ Mean of duplicates (range).

An obvious question arises. Is the effect of activated T cell factor(s) limited to the functionally specialised immunocompetent B cells, or could it also stimulate other bone marrow-derived cells and bone marrow cells? Precursors of antibody-forming cells represent only part of the bone marrow population; another large portion comprises undifferentiated stem cells, or cells engaged in haemopoiesis (erythropoiesis and granulopoiesis).

The answer was sought as follows. Activated T cells were obtained from cultures of spleen cells incubated with phytohaemagglutinin (PHA). The stimulatory effect of PHA was measured by pulse labelling with ³H-thymidine. Haemopoietic stem cells were enumerated by the colony-forming unit (c.f.u.) assay¹¹. A sample of the cultivated cell suspension in which the stem cells (c.f.u.) were to be detected, was injected into lethally irradiated syngeneic mice. Spleens of recipients were removed 8 d later and macroscopic colonies on their surface were counted. Each colony is a clone arising from a single pluripotent haemopoietic stem cell and contains cells of the same morphological character, either erythrocytic, megakaryocytic or granulocytic^{11–14}. No lymphocytic colonies have been reported so far, even in spleens of mice that received cells from PHA-stimulated donors¹⁵. Colony-forming stem cells can be obtained from bone marrow, spleen, blood and lymph nodes (in decreasing order of frequency) but not from thymus or thoracic duct cells^{15–18}.

In my first experiment, normal spleen cells were cultivated *in vitro* for 3 d with or without PHA (2.5 µg ml⁻¹) and c.f.u. in the cultures was counted by the transfer of 10⁶ cells to irradiated recipients. To verify that the tissue culture reflected the effect of PHA *in vivo*¹⁵ some irradiated recipients were given freshly isolated spleen cells (10⁵ to 10⁶) of either normal donors or donors injected with PHA 1 d before the transfer. Table 1 shows that stimulation of spleen cells with PHA, either *in vivo* or *in vitro*, increased the number of haemopoietic cells (c.f.u.) about three times ($P < 0.01$, by Wilcoxon's unpaired rank test, ref. 19).

The following experiments were performed to determine if the effect of PHA on stem cells is mediated by T cells. Spleen cells were treated either with anti-theta serum plus complement to eliminate T cells, or with normal serum and complement as a control. The cells were washed and cultured for 3 d with or without PHA. c.f.u. were enumerated as previously. Table 2 shows that the stimulation of DNA synthesis and the increase of stem cells (c.f.u.) observed in control cultures (containing T cells) ($P < 0.01$) did not occur in experimental cultures in which T cells were eliminated ($P > 0.05$).

To show that the effect of T cells on c.f.u. is mediated by a factor(s), supernatant fluids from previously described cultures were collected, filtered through 0.45 µm Millipore filters and stored frozen at -20° C. They were then tested

TABLE 2 Role of theta-positive cells (T cells) in increase of splenic colony-forming cells in cultures stimulated with PHA

Cell treatment before culture	PHA*	Recipient mice No. cells† injected, × 10 ⁶	c.f.u. per spleen‡, individual‡	c.f.u. per 10 ⁶ injected cells	³ H-thymidine pulse§ (d.p.m. per 10 ⁶ cells)
Normal serum + C'	—	9.0	0, 1, 1, 1, 1, 2, 2, 2	1.4 (<1–2)¶	2,955 (2,850–3,080)
	+	4.5	1, 1, 3, 3, 3, 4, 5	6.3 (2–11)	103,750 (103,500–104,000)
Anti-theta serum + C'	—	7.2	2, 3, 3, 3, 4, 5, 7, 9, 9	6.9 (3–12)	2,460 (2,360–2,560)
	+	6.3	2, 3, 3, 5, 5, 9	7.1 (3–14)	2,310 (1,830–2,790)

Anti-theta serum was prepared by AKR mice given ten weekly injections of C3H thymocytes (10⁷ cells per injection, intraperitoneally). 100 µg of a pertussis vaccine (gift from the Massachusetts State Laboratory) was given with the eighth injection. The serum was specific for thymus cells (titre 1:8,000) and killed 40–50% of spleen cells as determined by trypan blue exclusion. Normal guinea pig serum absorbed twice with mouse spleen and liver cells (5:1, v/v) was used as a source of complement (C'). Freshly isolated spleen cells were incubated, under sterile conditions, with anti-theta serum (5 × 10⁷ cells per ml of Puck's saline, final dilution of antiserum 1:10) in a water bath at 37°C for 20 min. Guinea pig serum was then added as a source of complement (1:10) and left for another 20 min. Normal mouse serum (absorbed) served as a control. Cells were washed in Puck's saline and cultivated for 3 d as described in Table 1. As anticipated, specific killing of T cells resulted in a relative increase of c.f.u. per 10⁶ viable cells remaining in the spleen cell suspension. The increase was expected to be two-fold but was actually higher (compare first and third row in Table 2). The possible stimulating effect of antigen-antibody complex on CFU might be considered.

Footnotes are explained in the legend to Table 1.

TABLE 3 Stimulation of bone marrow c.f.u. with a T cell product *in vitro*

Experiment	Culture medium	Recipients		c.f.u. per 10 ⁶ injected cells	³ H-thymidine pulse§ (d.p.m. per 10 ⁶ cells)
		No. cells† injected, × 10 ⁵	c.f.u. per spleen, individual‡		
A	MEM	4.3	3,3,4,4,5,6,6	10.0 (7-14)	10,500 (9,600-11,500)
	MEM + PHA	4.5	4,4,4,5,5,5,6,6	11.0 (9-13)	7,500 (7,500-7,500)
	S _{MEM} 1:20	8.4	4,5,6,6	6.4 (5-7)	12,300 (11,200-13,400)
		16.8	9,9,10		
	S _{PHA} 1:20	4.0	6,7,8,10		
B		8.0	14,14,16,19	20.0 (15-24)	31,450 (31,300-31,600)
	S _{MEM} 1:50	2.5	1,2,2,3,3,4	10.0 (4-16)	12,950 (11,200-14,700)
	S _{PHA} 1:50	2.5	10,10,12,14,15,18	53.0 (40-72)	31,000 (28,000-34,000)
	1:100	2.5	3,4,4,5,6,9	21.0 (12-36)	27,400 (24,000-31,000)
	S _{anti-theta, MEM}	2.5	3,4,4,4,5	16.0 (12-20)	15,500 (13,000-18,000)
	S _{anti-theta, PHA}	2.5	2,2,2,3,3,4,4,5,5	14.0 (8-20)	18,000 (16,000-22,000)

Bone marrow cells (pooled from several donors) were flushed from femurs with cold Puck's saline, washed and resuspended in the culture medium as described in Table 1. Supernatant fluids added to the medium at various concentrations were collected, in previous experiments, from 3-d cultures of spleen cells in the medium without (S_{MEM}) or with (S_{PHA}) PHA. Supernatants added in experiments A and B came from two different experiments in which the stimulating effect of PHA on splenic c.f.u. in the cultures was determined. 'S_{anti-theta, MEM}' and 'S_{anti-theta, PHA}' are supernatants from cultures shown in Table 2 (third and fourth row) in which spleen cells were pretreated with anti-theta serum plus C' and then cultured with or without PHA in the medium. Splenic B cells (obtained by treatment of spleen cell suspension with anti-theta serum and complement before cultivation) were also used as the target for the factor. Results (unpublished) were comparable to those reported here with bone marrow cells.

MEM, minimal essential medium.

Footnotes are explained in the legend to Table 1.

for the presence of a cell-free substance able to stimulate haemopoietic stem cells *in vitro*. Bone marrow cells (which do not respond to PHA), rather than spleen cells, were the source of c.f.u. and the target for the hypothetical factor. This eliminated the possibility of stimulation of T cells by trace amounts of PHA in the supernatant fluids. Bone marrow cells were flushed from femurs of normal donors, washed and resuspended in the culture medium containing various concentrations of either control supernatants from normal, untreated spleen cultures (S_{MEM}) or active supernatants from cells cultured with PHA (S_{PHA}). Another set of control cultures included bone marrow cells with or without PHA in the medium. Cells were tested for the number of c.f.u. after 24 h of cultivation. Some culture tubes were kept for 3 d and were pulse-labelled with ³H-thymidine.

PHA failed to stimulate DNA synthesis and c.f.u. ($P > 0.05$) in bone marrow cell cultures (Table 3, experiment A). In contrast, the presence of S_{PHA} in the culture medium increased DNA synthesis in cultures and increased the number of active c.f.u. two to three times compared with control cultures containing S_{MEM} (experiments A and B). The factor was active up to a dilution of 1:100 in the medium with an optimum concentration of about 1:50. Supernatant fluid from spleen cells pre-treated with anti-theta serum plus complement and then cultured with PHA did not contain any stimulatory activity ($P > 0.05$).

It can be stated that mouse splenic T cells activated with PHA *in vitro* release a substance(s) which can stimulate proliferation of bone marrow cells and activate haemopoietic stem cells *in vitro* and *in vivo*. The possibility of such a mediator was implied by previous observations that the administration of thymus-dependent antigens changed considerably the frequency of haemopoietic stem cells in blood and spleen²⁰⁻²¹. A similar effect was observed by Micklem¹⁵ after *in vivo* injection of PHA, a specific mitogen of mouse T cells²²⁻²⁵. My results provide direct proof of existence of such a factor(s).

The chemical nature of this T cell product is not known. I suggest, however, that it is related to immunoregulating factors induced in T cells by antigen⁶⁻⁹ rather than to 'thymosine', a soluble substance obtained from normal thymus cells and able to promote immunological maturation of B cells²⁶⁻³². The suggestion is based on the observation that the factor described here can be obtained from stimulated peripheral T cells only. Thymus cells, either normal or incubated with PHA, do not release an active substance that has this effect on stem cells (unpublished results of E. B. Waner and myself).

If upheld, my observations will offer a mechanism by which activated T cells can regulate not only antibody formation in specialised immunocompetent cells, as described by others, but also differentiation of more primitive stem cells and haemopoietic cells in bone marrow. This model will provide another test for comparative analysis of biological functions of humoral products from T cells.

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Animal longevity and protein turnover rate

THE life spans of various mammalian species differ but the biochemical mechanism of this regularity is unknown. In our

TABLE 2 Turnover of homologous proteins of various species labelled with ¹³¹I (ref. 1, column reference indicates 262 and 263).

Species	Albumins		γ globulin		Maximum lifespan (yr)
	Time interval (d)	Half-life (d)	Time interval (d)	Half-life (d)	
Human	7-28	15.0	4-21	13.1	110-115
Cow	2-30	20.7	6-27	21.2	20*
Monkey			4-24	6.6	30
Dog	7-28	8.2	7-17	8.0	18
Rabbit	4-22	5.7	4-21	4.6	15
Guinea pig			4-13	5.4	7
Mouse	3-7	1.2	1-5	1.9	3
Rabbit		4.5			5

* These data apply to ruminants and herbivores in general.

turnover, and the turnover is lower for dogs, and still lower for cows, monkeys and human beings.

In terms of a life span there is a negative correlation between the longevity of animals and their protein turnover rate. The existence of such a regularity has attracted no attention so far. The protein turnover rate, however, does

TABLE 1 Turnover of plasma proteins of various species of mammals.

Protein	Species	Active matter	Method*	Half-life (d)	Reference to literature†	Maximum life-span (yr)
General plasma protein	Rat	³⁵ S-DL-methionine	D	2.6	207	5
		³⁵ S-DL-methionine	D	4.4	222	
		DL-serine-3	H	3.0	232	
	Dog	Rhodospirillum-rubrum- ¹⁴ C	H	3.0	198	18
		³⁵ S-L-methionine	D	3.5	259	
		³⁵ S-DL-methionine	D	5.2-6.4	115	
		¹⁵ N-DL-lysine	H	5.4	230	
		DL-lysine-6	H	5.0	208	
		³⁵ S-DL-methionine	H	5.0	115	
		³⁵ S-DL-methionine	D	6.6	260	
		³⁵ S-DL-methionine	D	9.2	115	
		¹⁵ N-glycine	D	10.0	261	
		¹⁵ N-glycine	D	7.0	240	
Albumin	Human (male)	¹⁵ N-glycine	D	9.0	240	110-115
	Human (female)	¹⁵ N-glycine	D	9.0	240	
	Rat	³⁵ S-L-methionine	D	5.0	259	
	Dog	DL-lysine-6	H	6.9	208	
	Human (male)	¹⁵ N-glycine	D	20	240	
	Human (female)	¹⁵ N-glycine	D	20	240	
Globulin	Rat	³⁵ S-L-methionine	D	3.0	259	240
		DL-lysine-6	H	3.3	207	
	Dog	³⁵ S-L-methionine	D	3.0	259	
		³⁵ S-L-methionine	D	2.0	259	
	Rat	³⁵ S-L-methionine	D	3.0	259	
		³⁵ S-L-methionine	D	3.2	259	
	Human (male)	¹⁵ N-glycine	D	12	240	
		¹⁵ N-glycine	D	19	240	
	Human (female)	¹⁵ N-glycine	D	9.0	240	
		¹⁵ N-glycine	D	18	240	
α ₁ globulin	Human (male)	³⁵ S	D, CP	4.2	227	227
α ₂ globulin	Human (female)	³⁵ S	D	8.1	227	

* "D", is a direct method used to determine the isotope concentration in protein at some intervals after injection of the labelled material. "H" is (Hevesi's method) introduction of the labelled protein to the recipient animal with subsequent measurement. "CP" is the constant fund method. Half-life span measured by the different methods is approximately the same.

† Column reference indices Sam Tarver¹.

opinion there is a sufficient amount of experimental data to solve this problem. We refer to the data presented in twelve investigations by different authors collated into one table (Table 1), and the data of the two works collated in another (Table 2)¹. In these, the data on the protein plasma turnover for various species of mammals is presented. We have added a column on the maximum life span of the species² into each table.

Table 1 shows that the highest rate of protein turnover is observed in the rat, it is lower in the dog, and still lower in human beings. Table 2 shows that the small animals, such as mice, guinea pigs or rabbits, have the highest rate of

characterise the intensity of protein synthesis, and this in turn, depends on the intensity of DNA activity.

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Solubilisation of angiotensin II receptors in rabbit aortae membranes

HORMONE-RECEPTOR interaction is the first step of a chain of cellular processes leading to the hormonal response. Receptor sites are believed to be macromolecular components finite in number, possessing a genetically determined structure and capable of specific recognition and interaction with hormones with a high degree of specificity and affinity. Receptors of steroid hormones are intracellular, whereas those of neurotransmitters and polypeptide hormones are located in the cell membranes¹.

Angiotensin II is a polypeptide hormone which has, among many pharmacological actions, the basic property to contract smooth muscles². The availability of a ³H-angiotensin II having a high specific activity and possessing the full biological activity of the native hormone³ allowed us to demonstrate the presence of angiotensin receptors in the intact rabbit aorta⁴, in the microsomal membranes extracted from homogenates of intimal medial layers of rabbit aortae⁵, and in a membrane fraction derived from the plasma membrane⁶. At the various levels of purification the binding sites were considered as hormone receptors, as the kinetic constants of angiotensin binding were identical to those of the biological responses induced by the hormone.

Here we describe the solubilisation of the above described angiotensin-binding components of smooth muscle cells of rabbit aorta, and provide some information about the molecular conformation of angiotensin required for its interaction with receptors.

The microsomal membranes were extracted from homogenates of intima media of rabbit aortae by differential centrifugation⁷. These membranes were considered to be essentially derived from smooth muscle cells because the contribution of intima was less than 1% total mass. Receptors were solubilised by suspending the 100,000g microsomal pellet in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl and deoxycholate (7-deoxycholic acid, Sigma; final concentration 0.2–1.0%). The membrane protein concentration⁸ was 4.6 mg ml⁻¹. After a 30 min incubation at 0°C, the membrane suspensions were centrifuged for 1 h at 200,000g and the resulting soluble supernatants were assayed for angiotensin binding. After incubation of soluble material with ³H-angiotensin II (specific activity 40 Ci mmol⁻¹), aliquots

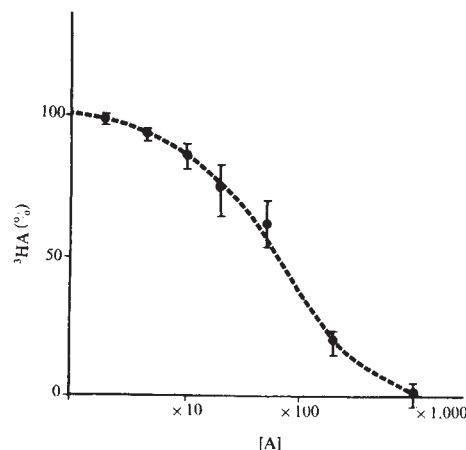


Fig. 2 Inhibitory effect of unlabelled angiotensin II (A) on the binding of 2.5×10^{-8} M of ³H-angiotensin II to solubilised material. The various concentrations of unlabelled angiotensin II are indicated on the abscissa (10, 100 and 1000-fold excess compared to the ³H-angiotensin II concentration). ³H-angiotensin II and unlabelled angiotensin II were added simultaneously to the incubation mixture. The nonspecific binding (40% of bound radioactivity) was subtracted from each experimental value. $\bar{x}$, the mean and s.d. of 2–15 experiments.

(400 μ l) of reaction mixture were placed on the surface of G-50 fine Sephadex columns (14.0 $\times$ 0.6 cm) equilibrated and run at 4°C with 50 mM Tris-HCl buffer pH 8.0 with or without deoxycholate. The eluates were added to scintillation fluid and counted in a liquid scintillation spectrometer. The validity of Sephadex for the separation of bound and free ³H-angiotensin II was demonstrated in control experiments performed with buffer alone, showing that only 0.01% of ³H-angiotensin II appeared in the void volume. Each experimental sample was corrected for the radioactivity appearing in the void volume in the control.

Treatment of microsomal membranes by deoxycholate solubilised 50% of membrane proteins and solubilisation was maximal at concentrations of deoxycholate greater than 0.2%. After treatment with deoxycholate 80% of the specific binding of ³H-angiotensin II was recovered in the soluble supernatant and 20% in the pellet. The binding of ³H-angiotensin to the solubilised material is proportional to the concentration of protein. Time course studies and concentration-dependent binding curves of ³H-angiotensin II were performed in the presence and absence of 1000-fold excess of unlabelled angiotensin II. The specific binding is considered as the difference between the binding of ³H-angiotensin II measured in the absence of unlabelled angiotensin.

The specific binding of ³H-angiotensin II to the solubilised material reached equilibrium within 5 min at 29°C (Fig. 1). No significant specific binding was observed after 90 min of incubation at 4°C; in contrast the non specific binding was minimally affected by changes in temperature. The association rate constant at 29°C is $K_1 = 2.5 \times 10^5$ mol⁻¹ s⁻¹. Dissociation of angiotensin-receptor complex was studied by adding 1000-fold excess native angiotensin to the samples preincubated for 5 min at 29°C with 2.5×10^{-8} M of ³H-angiotensin II. The samples were then incubated for varying periods before being assayed for specific angiotensin binding. During the first 2 min dissociation of the ³H-angiotensin binding was a first order kinetic reaction, the rate constant of which being $K_{-1} = 4.1 \times 10^{-3}$ s⁻¹ (Fig. 1). A Scatchard plot⁹ of the specific binding of ³H-angiotensin II to the deoxycholate-solubilised proteins indicates that a single high affinity receptor site is apparently being measured with ³H-angiotensin II concentrations varying from 2.5×10^{-9} M to 10^{-7} M. The number of specific binding sites is 2 pmol per mg of protein, and the apparent dissociation constant

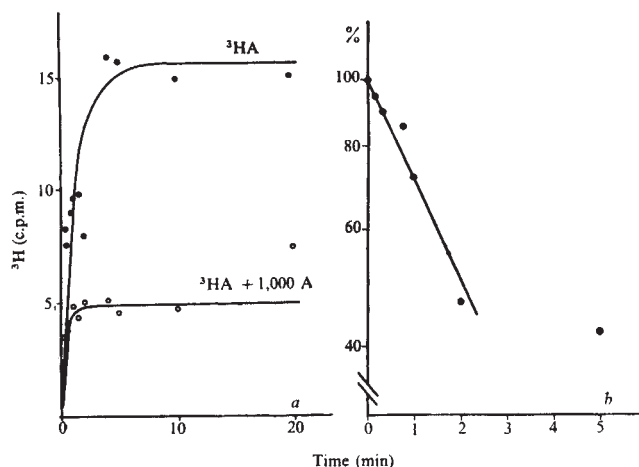


Fig. 1 a, Time course of binding of 2.5×10^{-8} M of ³H-angiotensin II to solubilised material at 29°C in the absence (³HA) and presence of 1000-fold excess of unlabelled angiotensin (³HA + 1000 A). Results expressed as c.p.m. mg⁻¹ ($\times 10^3$); b, displacement of bound radioactivity induced by addition of 1000-fold excess of unlabelled angiotensin to solubilised material after its incubation with 2.5×10^{-8} M of ³H-angiotensin II for 5 min at 29°C. Results are expressed in % of bound radioactivity.

TABLE 1 Inhibitory effect of angiotensin II, angiotensin analogues and fragments, and L-noradrenaline on ³H-angiotensin II specific binding to deoxycholate-solubilised material

	Membranes	Deoxycholate-solubilised material
1 2 3 4 5 6 7 8 NH ₂ -Asp-Arg-Val-Tyr-Val-His-Pro-Phe-COOH	100	100
—————		45
—————		0
—————	81	100
—————	95	100
—————	39	30
—————	34	50
—————	4	35
—————Phe—————	92	85
—————Ile—————	43	90
L-noradrenaline	0	0
	0	0

The concentrations of ³H-angiotensin II and other drugs were 2.5×10^{-7} M and 5×10^{-7} M, respectively. Conditions of incubation and expressions of results are as in Fig. 2. For each compound, six determinations of ability to compete were made. The elucidation of the structure of angiotensin derivatives has been detailed^{15,16}. Data obtained on membranes are from ref. 6.

calculated from the slope of the plot is K_d 29°C $\sim 2.0 \times 10^{-8}$ M. The specificity of the angiotensin binding to the solubilised material was assessed by displacement experiments shown in Fig. 2 and Table 1.

The membraneous hormone receptors which have already been solubilised and partially characterised are those for insulin in liver and fat cells¹⁰, for catecholamines in myocardium¹¹ and turkey erythrocytes¹², and for acetylcholine in the electric tissue of fish¹³. That the angiotensin receptors of the rabbit aorta smooth muscle membranes were solubilised as described here is supported by several lines of evidence: first, the kinetic constants of ³H-angiotensin II binding obtained in our previous studies on intact aorta and microsomal membranes are similar to those measured on solubilised material (Table 2). These kinetic constants are very close to the kinetic constants of the biological response elicited by angiotensin, that is contraction of intact aorta⁴ and release of calcium from microsomes^{5,14}. It seems therefore that the binding sites of angiotensin may be considered as the major elements of the receptors responsible for the induction of the biological response. The binding capacity of the solubilised material is lower than that previously measured on microsomal membranes (Table 2). This is partially explained by the dissociation of the angiotensin-receptor complexes which occurs during chromatography on Sephadex: the amount of bound radioactivity was reduced two-fold by an additional gel filtration. Second, only the specific binding of angiotensin to solubilised material was temperature dependent, indicating that the specific binding process possibly involves a variation of conformation of the ligand and/or the binder, and also indicates the possible participation of hydrophobic groups in the binding process.

Third, solubilised receptor-angiotensin interaction is specific; ³H-angiotensin II binding was only inhibited by native angiotensin II and some angiotensin derivatives. As shown in Table 1 the 2-8 and 3-8 angiotensin fragments had an

inhibitory effect on ³H-angiotensin II binding identical to that of the unlabelled angiotensin II; the 1-7, 4-8, 5-8 and 6-8 fragments had a partial inhibitory effect; the 1-6 hexapeptide, which has no biological activity, had no effect. The absence of inhibitory effect to the 1-6 fragment is a strong argument for the specificity of the binding as it shows that the binding of angiotensin to the solubilised material is not related to a nonspecific interaction between some hydrophobic part of the angiotensin molecule and deoxycholate molecules which may remain associated to any of the solubilised membrane proteins.

These results agree with those previously observed on membranes⁶. Fermandjian *et al.*^{15,16} have demonstrated that the organisation of the angiotensin molecule in concentrated aqueous solutions and polar organic solvents, such as trifluoroethanol, was that of a cross- β type defined by two turns involving the 3, 4, 5 and 6, 7, 8 amino acids respectively, which was illustrated in our previous publication⁶. The present results indicate that binding to receptor sites does not depend upon the N-terminal as long as the amino acids responsible for the two turns are present. These results also indicate that the C-terminal turn alone allows a partial binding. It seems therefore that the interaction of the peptide with its receptor site requires its organisation in a cross- β conformation which has been shown to stem essentially from the His-Pro sequence (D. Greff, S. F., P. F., M. C. Khosla, R. R. Smeby, and F. M. B., unpublished). These considerations illustrate the extreme similarities of the interactions existing between angiotensin and the solvents which have been used in the physical studies, and those occurring between angiotensin and receptor sites. This demonstrates the validity of the angiotensin model established for such solvents. In addition to the cross- β organisation the Phe⁸ is of major importance in the binding process: binding is halved by its removal. The maintenance of full competitive activity with ³H-angiotensin binding when Ile⁸ replaces Phe⁸ may be ex-

TABLE 2 Comparison of the kinetics of ³H-angiotensin II binding to deoxycholate-solubilised receptor, microsomal membranes and intact aorta

	Aorta	Microsomal membranes	Deoxycholate-solubilised material
Specific binding capacity	0.8×10^{-14} mol mg ⁻¹	3.5×10^{-11} mol mg ⁻¹	2.0×10^{-12} mol mg ⁻¹
Dissociation constant	1.3×10^{-8} M	5.5×10^{-8} M	* 2.0×10^{-8} M † 1.6×10^{-8} M
Biological response (K_m)	‡ 1.5×10^{-8} M	§ 2.3×10^{-8} M	

* From Scatchard plots

† From K_{-1}/K_1

‡ Contraction

§ Ca²⁺ release.

Results on aorta and microsomal membranes are from ref 4 and 5.

plained by the similar hydrophobic properties of both terminal amino acids. In solution, however, the intramolecular interactions promoted by Phe⁸ and Ile⁸ respectively are somewhat different as observed by circular dichroism (D. Greff, S. F., P. F., M. C. Khosla, R. R. Smeby, and F. M. B., unpublished) which underlines the delicate variations of angiotensin II conformation with the environment.

The comparison of the inhibitory effects of the various angiotensin derivatives on the ³H-angiotensin II binding with their biological activities is difficult because only some of them were tested on isolated rabbit aorta¹⁷. The marked inhibitory effect on ³H-angiotensin II binding of the competitive antagonist (Ile⁸)-angiotensin II agrees with its high affinity for receptor sites of rabbit aorta¹⁸. The inhibitory effects on ³H-angiotensin II binding of the 1-7, 2-8, 3-8 fragments and of the Phe⁴ analogue was expected on the basis that they all have some intrinsic contractile activity. At least for these latter two peptides, whose activities were measured on rabbit aorta¹⁹, it seems that their affinity to receptors as measured in our study is higher than that expected from their contractile effect. This was also observed on isolated membranes⁶. A discrepancy between the binding to receptor sites and the contractile response might be easily explained if the biological response is considered as a complex phenomenon depending both on a good adjustment of angiotension to receptor 'cavity' (which would be, as discussed above, ensured by its cross- β organisation) and the interaction of specialised groups of the molecule with membrane components which would trigger the biological response. This discrepancy might also indicate a great complexity of the receptor site. One might speculate that the binding involves more than one macromolecule and the part concentrated in this preparation is specific for the C-terminal sequence of angiotensin II. It is interesting in this respect to note that in the case of catecholamines the part of the molecule responsible for binding was clearly distinguished from that responsible for the activation of adenylyl cyclase²⁰.

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Electrical coupling between afferent nerve terminal and intrafusal muscle fibre in the frog muscle spindle

FROM electronmicroscopic observations, Katz¹ has pointed out the existence of junctions between the afferent nerve terminal bulbs and the intrafusal muscle fibres in frog muscle spindle. It was suggested that the junction might play an important part in the transducing function of the receptor. Here we give physiological evidence for such a role in the simple-type spindle receptors.

Muscle spindles with intact afferent innervation were isolated from the sartorius muscles of Japanese frogs (*Rana nigromaculata*). The isolated spindle was placed at its *in situ* length in a pool of Ringer's solution (111.3 mM NaCl, 1.88 mM KCl, 1.08 mM CaCl₂ and 2.38 mM NaHCO₃) on a glass plate. The nerve fibre (2-3 cm) was passed into another Ringer's pool through a liquid paraffin pool. The two Ringer's pools were separated by an embankment of vaseline, which in turn was interrupted at its centre by the 1 mm wide paraffin pool. The pools of Ringer's solution were connected to calomel electrodes through Ringer-agar bridges. The potential between a microelectrode, used for recording from the spindle, and the calomel electrode of the same Ringer's pool, was led to a high input-impedance amplifier and displayed on one beam of a dual beam oscilloscope. The potential across the paraffin gap was fed into a high input-impedance differential amplifier and displayed on the other beam of the oscilloscope. Glass microelectrodes (resistance 20-50 M Ω , filled with 3 M KCl or 2 M NaCl) were manipulated under visual control, using an inverted microscope. Constant current stimuli (pulses of 0.05 ms duration or direct current) were given extracellularly to the axon terminal or intracellularly to the intrafusal muscle fibre.

The simple type spindles which were used had normal innervation: the parent axon divides into two myelinated branches; one of them again subdivides into two short myelinated branches, while the other does not². Our experiments concern only the non-subdivided branch and the intrafusal muscle fibre innervated by it. Propagated and abortive spike discharges were defined, as usual^{3,4}, by recording across the paraffin gap.

Spontaneous spike discharges were recorded extracellularly by a microelectrode in contact with the surface of the nerve terminal. The amplitude was about 1 mV (negative). As the microelectrode was moved away from the nerve terminal in

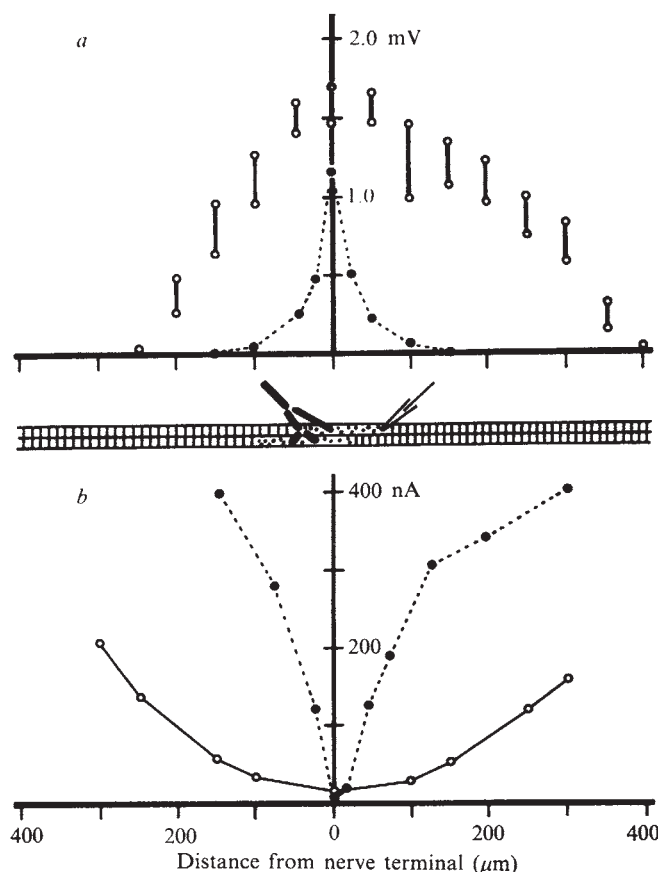


FIG. 1 Decrement of electrical events with increasing distance from nerve terminal. Glass microelectrodes for recording (a) and stimulating (b) were moved along the longitudinal axis of the intrafusal muscle fibre. —, Intracellular results; ----, extracellular results. Centre panel shows reticular area (stippled) and polar regions (striated), drawn to scale. a, Amplitude of fast potentials recorded from muscle, coincident with spontaneous propagated spikes. Microelectrodes were filled with 3 M KCl for intracellular recording and 2 M NaCl for extracellular recording. b, Current strength necessary to excite nerve terminal. Negative pulses were given extra- and intracellularly through glass microelectrodes. Efficacy of positive pulses (data not shown) was about the same.

either direction along the intrafusal muscle fibre, the spike amplitude decreased nearly exponentially, falling to 50% at a distance of 20 μm , and becoming undetectable at a distance of 150 μm from the terminal.

Insertion of the microelectrode into the reticular zone of the intrafusal muscle fibre revealed a resting potential of -50 to -60 mV, and negative spikes which were coincident with the propagated and abortive spikes. These spikes could be recorded intracellularly (solid vertical bars in Fig. 1a) at distances as great as 400 μm from the nerve terminal; by that point well-developed striations were present.

These results indicate the existence of a strong coupling between the axon and the intrafusal muscle fibre, allowing fast electrical changes to pass through easily. This coupling seems to have a capacitive component because the above mentioned spikes were transformed as if differentiated (data not shown). Such coupling was also indicated by the following experiments.

Electrical pulses were applied at various points along the surface of the muscle fibre through a microelectrode, and the current intensity necessary to excite the terminal was measured. The dotted line in Fig. 1b demonstrates that a negative current pulse of about 2 nA was required at the level of the nerve terminal, and that the current requirement increased steeply with lateral displacement of the stimulating electrode in either direction.

The same experiment was then conducted following penetration of the muscle fibre by the stimulating electrode (solid line in Fig. 1b). All stimuli were subthreshold for muscle twitching. When a negative pulse of 3 nA was applied at the level of the nerve terminal, a propagated spike response did occur along the parent axon. No muscle twitching was observed, of course. In sharp contrast to the extracellular stimulation, the current necessary to stimulate the nerve terminal increased only slightly over a longitudinal distance of 100 μm . Even at a distance of 300 μm from the terminal, a pulse of only 150 nA was still sufficient to excite the terminal. Electrical pulses of 100–200 nA, applied intracellularly to the polar region of the intrafusal muscle fibre, occasionally provoked muscle impulses and twitching.

Depolarisation of the intrafusal muscle fibre by means of current injected through an intracellular microelectrode increased the rate of spontaneous afferent discharge, and hyperpolarisation decreased it (Fig. 2). A quasilinear relationship between the current and the discharge rate was obtained in the range of $+20$ to -20 nA, during which no contraction could be observed. This suggests that the axon terminal may be coupled with the intrafusal muscle fibre by a relatively low resistance.

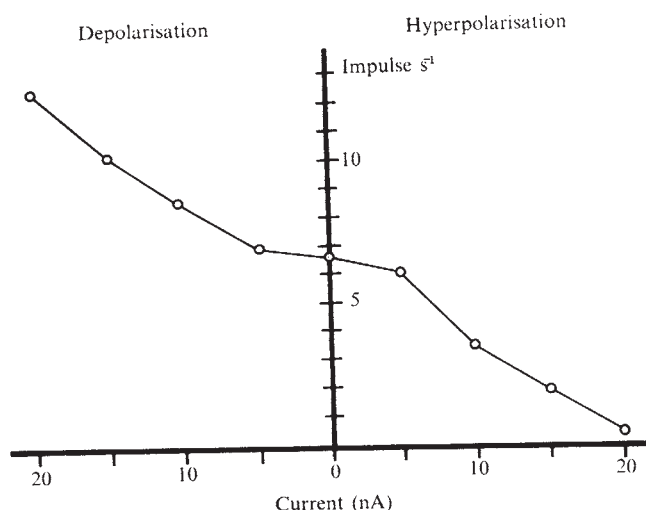


FIG. 2 Effect of direct current injection into muscle fibre upon propagated spike discharge rate of afferent nerve. The microelectrode was inserted into intrafusal muscle fibre 50 μm from nerve terminal.

In the muscle spindle, the afferent nerve terminal and the intrafusal muscle fibre may have electrical coupling through capacitance and resistance, arranged in parallel. Such an electrical coupling must contribute to the transmission of events from the axon terminal to the intrafusal muscle fibre and *vice versa*. It may play an important role in the transduction mechanism of the receptor. It is likely that the sensory nerve terminal is also activated by conducted muscle fibre impulses; it has been found that these 'early discharges' in turn precede the twitch of the intrafusal muscle fibre in mammalian spindles^{5,6}.

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Mouse brain antigen detected by rat anti-C1300 antiserum

THE concept of the cell surface mediating specific cell-cell interactions is pertinent to neurogenesis in which the complex cell interactions known to occur may involve the recognition of specific cell surface components. Characterisation of neuronal cell surfaces is necessary to prove this idea and to elucidate functional mechanisms. Antisera raised against lymphoid cells have provided valuable information on the membrane constituents of lymphoid cells¹⁻³, and so I have used a similar approach to identify antigenic components expressed on cells of the nervous system of the mouse. The murine

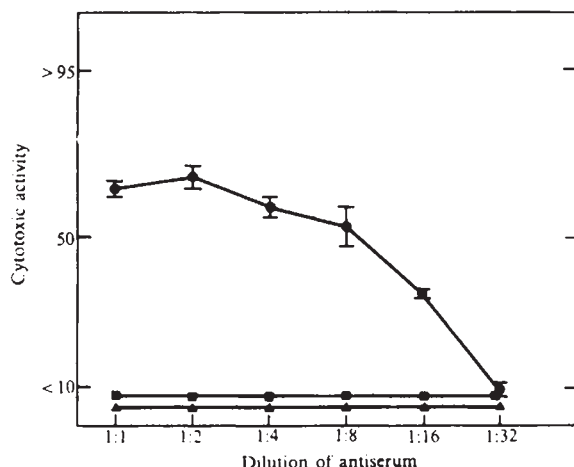


Fig. 1 Titration of rat anti-C1300 antiserum against C1300 tumour cells (●), lymph node cells (LNC) of A mice (■), or LNC of C57 mice (▲). To label the target cells, 0.5 ml of a cell suspension containing 5×10^7 cells ml^{-1} was incubated for 30 min at 37° C with 50 μCi of ^{51}Cr (Na_2CrO_4 , Amersham) in medium containing 10% foetal calf serum (FCS). The cells were washed twice and resuspended to 3×10^6 cells ml^{-1} in medium containing FCS. A volume of 20 μl of cell suspension was incubated at room temperature for 30 min with 20 μl of various dilutions of antiserum. The cells were washed with 300 μl of medium and, after centrifugation at 800 r.p.m. for 7 min, the cells were resuspended in 50 μl of a 1:4 dilution of rabbit serum and incubated at room temperature for 30 min. Medium (150 μl) was added and the tubes centrifuged at 1,000 r.p.m. for 10 min. The radioactivity in 100 μl of supernatant from cells exposed to antibody and complement (ab) was measured and compared with that released from cells exposed to detergent (max) or complement alone (comp). Cytotoxic activity was calculated by the formula

$$(\text{ab} - \text{comp})/(\text{max} - \text{comp}) \times 100.$$

In all experiments the absolute release of ^{51}Cr caused by complement was less than 10% of the maximum release. The antiserum tested at a 1:2 dilution in the absence of complement was not toxic. Duplicate or triplicate determinations were made in all cytotoxicity assays.

Although the plateau of maximum cytotoxic activity observed in the ^{51}Cr release cytotoxicity assay was 60% of the amount of ^{51}Cr released by detergent, trypan blue analysis showed that 90%-94% of viable ascites cells were killed by dilutions of antiserum up to 1:4.

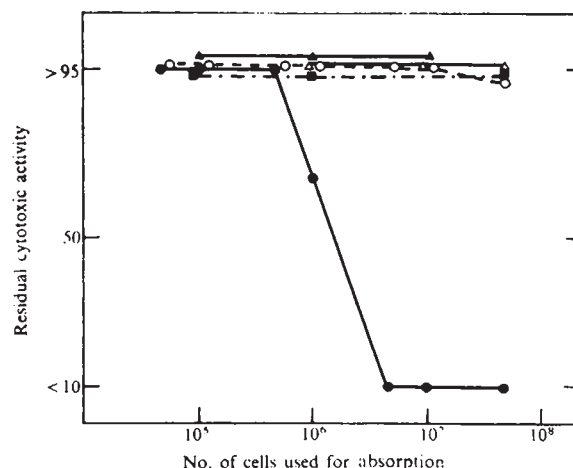


Fig. 2 Residual cytotoxic activity of rat anti-C1300 antiserum after absorption with C1300 ascites tumour cells (●—●), LNC of A mice (○—○), thymus cells of A (■—■), AKR (▲—▲), or 129 mice (△—△). Volumes (50 μl) of a 1:4 dilution of antiserum were incubated with varying numbers of cells for 30 min at room temperature. Volumes (20 μl) of antisera were assayed in duplicate as described in the legend to Fig. 1. The residual cytotoxic activity was calculated by the formula

$$(\% \text{ lysis by absorbed antiserum} / \% \text{ lysis by unabsorbed antiserum}) \times 100.$$

neuroblastoma C1300 can, in culture, give rise to cells which display many of the morphological, biochemical, and physiological properties characteristic of neurones⁴⁻⁹. Here, I present evidence indicating that rat antisera raised against C1300 tumour cells recognise a tissue antigen which is expressed predominantly on mouse brain.

The C1300 tumour is maintained in several laboratories by subcutaneous passage in strain A mice. For this study, the tumour was converted to an ascites form by repeated intraperitoneal passage of tumour cells into A strain mice, referred to subsequently as A mice. Two weeks after the intraperitoneal injection of 3×10^6 C1300 tumour cells, approximately 2×10^7 ascites cells could be obtained per mouse. The ascites cells used in these experiments were between 90% and 95% viable. Of the viable cells, more than 90% were C1300 tumour cells.

Antiserum was raised in adult Wistar rats by six intraperitoneal injections of 10^7 viable ascites tumour cells, at intervals of 14 days. The rats were bled 2 weeks after the last injection. Before absorption with mouse lymphoid cells the antiserum had a cytotoxic titre of 1:300 against both C1300 tumour cells and lymph node cells of A mice. The antiserum was absorbed five times for 30 min at room temperature with equal volumes of a mixture of spleen and thymus cells obtained from A, C3H/He, BALB/c, AKR, and C57BL (C57) mice. The absorbed antiserum was not cytotoxic for LNC of A or C57 mice. When tested against C1300 tumour cells, the antiserum had a cytotoxic titre of 1:16 (Fig. 1). In indirect immunofluorescence studies, using fluoresceinated sheep anti-rat immunoglobulin antibody, 93% of the viable ascites cells incubated with a 1:4 dilution of rat anti-C1300 antiserum demonstrated bright ring fluorescence. C1300 cells incubated with normal rat serum did not fluoresce. Less than 1% of LNC, thymus cells, or spleen cells of A mice were fluorescent after incubation with either rat anti-C1300 antiserum or normal rat serum. Aliquots of a 1:4 dilution of rat anti-C1300 antiserum were absorbed with varying numbers of C1300 ascites tumour cells, LNC of A mice or thymus cells of A, AKR, or 129 mice and tested for cytotoxic activity against C1300 tumour cells. A sample of 5×10^6 C1300 cells

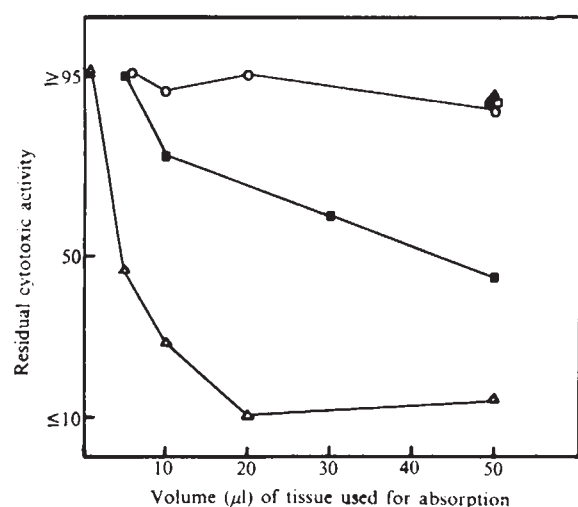


Fig. 3 Residual cytotoxic activity of rat anti-C1300 antiserum after absorption with various amounts of brain (Δ), kidney (■), liver (○), muscle (●), spleen (▲) and testes (□) of A mice. Volumes (50 µl) of a 1:4 dilution of antiserum were incubated with a measured volume of tissue at room temperature for 30 min. Volumes (20 µl) of absorbed antisera were assayed in duplicate as described in the legend to Fig. 1. The residual cytotoxic activity was calculated as described in the legend to Fig. 2.

removed all cytotoxic activity. No significant activity was removed by any of the other cell types (Fig. 2).

To investigate whether the antigen recognised by the rat anti-C1300 antiserum was a normal tissue antigen of A mice, aliquots of a 1:4 dilution of the antiserum were absorbed with various tissues of A mice and tested for residual cytotoxic activity against C1300 tumour cells. A putative anti-H-2^a antiserum raised in C57 mice by three intraperitoneal injections of 2×10^6 spleen cells of A mice was absorbed and tested in parallel. To control for dilution and possible non-specific absorption by the tissues, aliquots of the rat anti-C1300 and the C57 anti-A antisera were also absorbed with syngeneic rat tissues. The cytotoxic activity of the rat anti-C1300 antiserum was removed by brain and, to a lesser extent, by kidney of A mice. Brain tissue of A mice did not

absorb appreciable amounts of antibody activity from the anti-H-2^a antiserum. Liver, lung, spleen, and testes of A mice, could remove cytotoxic activity from the anti-H-2^a antiserum, but did not remove a significant amount of cytotoxic activity from the rat anti-C1300 antiserum. Absorption with muscle from A mice did not significantly lower the cytotoxicity of either antiserum (Table 1). In further experiments, aliquots of antiserum absorbed at a 1:4 dilution with either brain, kidney, liver, muscle, or spleen were tested for residual cytotoxic activity at 1:4, 1:8, 1:16, and 1:32. Compared with Wistar rat tissues, only brain and kidney of A mice removed significant amounts of cytotoxic activity from the antiserum. To estimate the relative amounts of the antigen expressed on brain and kidney of A mice, 50 µl volumes of a 1:4 dilution of the rat anti-C1300 antiserum were absorbed with various amounts of these tissues. The results of these experiments indicated that there was at least 10 times as much of the antigen on brain as on kidney of A mice. Liver, muscle, spleen, and testes did not absorb appreciable amounts of the cytotoxic activity (Fig. 3). The antigen recognised by

TABLE 1 Residual cytotoxicity of absorbed antiserum

Tissues used for absorption	Rat anti-C1300 antiserum		C57 anti-A antiserum	
	A (mouse) tissues	Wistar (rat) tissues	A (mouse) tissues	C57 (mouse) tissues
Brain	8	83	88	NT
Kidney	45	78	35	77
Liver	86	84	10	77
Lung	84	NT	30	96
Muscle	88	87	80	89
Spleen	89	88	0	NT
Testes	88	NT	58	80

Residual cytotoxic activity of rat anti-C1300 and C57 anti-A antisera absorbed with various tissues. Organs were removed from exsanguinated animals and chopped into pieces of roughly 1 mm³. The chopped tissues were pressed through a 333 mesh nylon net and the filtered suspensions washed four to seven times with medium containing 10% FCS. Diluted antiserum (50 µl) was absorbed with an equal volume of packed tissue for 30 min at room temperature. Volumes (20 µl) of absorbed antisera were tested as described in the legend to Fig. 1. The residual cytotoxic activity was calculated as described in the legend to Fig. 2. The titre of the C57 anti-A antiserum was 1:40 against both C1300 tumour cells and LNC of A mice. A 1:10 dilution of the antiserum was used in the absorption experiments. NT, not tested.

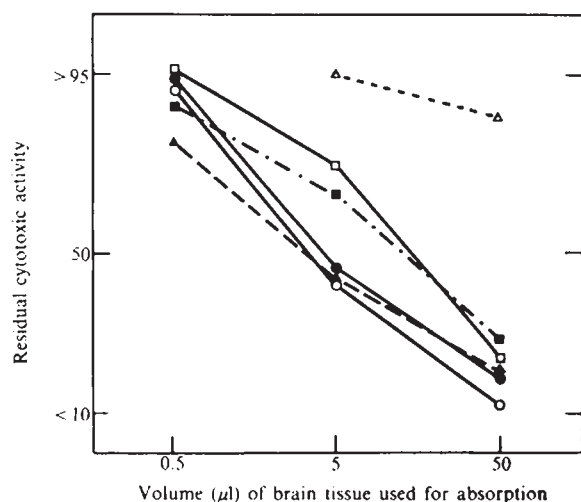


Fig. 4 Residual cytotoxic activity of rat anti-C1300 antiserum absorbed with various volumes of packed brain tissue of A (●—●), C57 (■—■), C3H/He (○—○), CBA (▲—▲), or DBA (□—□) mice or Wistar rats (Δ—Δ). The brain tissues were prepared as described in the legend to Table 1. Brains were removed from animals between 3 and 4 months of age. The residual cytotoxic activity was calculated as described in the legend to Fig. 2.

rat anti-C1300 antiserum was expressed in comparable amounts on the brains of A, C57, C3H/He, CBA, and DBA mice (Fig. 4).

C1300 tumour cells express several cell surface antigens present on brain as well as other tissues of certain mouse strains¹⁰. These antigens include H-2^a, the major histocompatibility antigen of A mice; the θ -C3H alloantigen; Pc-1, the plasma cell alloantigen; GCSA, the Gross cell surface antigen; Sk-1, the skin alloantigen; and the NZB autoantigen. None of these alloantigens is expressed predominantly on nervous tissue or present on the nervous tissue of all strains of mice. The inability of LNC or thymus cells of A mice to absorb the cytotoxic activity of the rat anti-C1300 antiserum indicates that the antigen recognised by the antiserum is neither H-2^a, θ -C3H nor the NZB autoantigen. The inability of thymus cells of AKR or 129 mice to absorb the cytotoxic activity of the antiserum eliminates the possibility that the antigen is either GCSA or GIX¹¹, Gross virus-related antigens. The antigen detected by the rat anti-C1300 antiserum is not Pc-1 since Pc-1 is present on liver and spleen as well as brain and kidney of A mice. Nor is the antiserum directed against the serologically defined Sk-1 antigen expressed on the brain tissue of A mice since a different Sk antigen is expressed on the brain tissue of C57

mice. In addition, Sk has not been reported to be present on kidney tissue. Thus, the antigen recognised by the rat anti-C1300 antiserum is a normal tissue antigen of A mice present on brain and, to a lesser extent, on kidney, but not on liver, lung, muscle, spleen or testes and distinct from the alloantigens previously determined to be present on both C1300 tumour cells and A brain. In subsequent communications, this antigen will be referred to as mouse brain antigen-1 (MBA-1).

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Flagellar rotation and the mechanism of bacterial motility

BACTERIAL flagella are generally composed of three morphologically distinguishable regions: (a) the long flagellar filament which accounts for more than 95% of the flagellar protein; (b) the hook, which is generally 80–90 nm long and has a characteristic shape, and (c) the basal structure which is composed of an intricate set of disks and rods attaching the hook to the cell membrane and cell wall^{1–3}.

Explanations of how the flagella move⁴ include the suggestion that helical waves are propagated along the filament^{5,6}, or that the filament behaves as a semirigid helical rotor^{4,7,8}. Berg and Anderson⁹ concluded that the evidence "... favours a model in which each filament rotates". The data presented here adds strong support to the existing evidence and provides a way to follow flagellar function that is independent of translational motility.

The methods that have been used to study motility depend on observation of the behaviour of the whole organism. This is a complex result of a series of events and is thus, at times, difficult to analyse and interpret. It would be useful to be able to follow the motion of a single flagellum or to assay for its activity in a way that does not depend on chemotaxis or cell motility. To do this, a series of experiments was designed based on the observation that bacteria can be found which appear to be 'tethered' by their flagellum to a particle

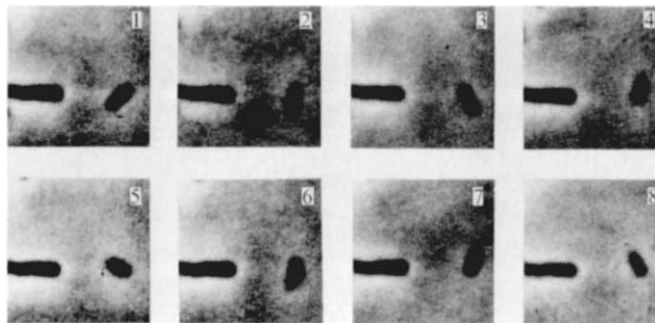


FIG. 1 The rotation of a cell bound to the microscope slide. *E. coli* strain MS1381 was grown on minimal medium with glycerol as the carbon source. Cells were put on a microscope slide and an equal volume of antipolyhook antibody diluted to 1:200 was added. After a 10 min lag, the cells began to spin. Their behaviour was recorded through a Zeiss phase contrast microscope onto video tape and then transferred to film. The pictures presented represent frames taken at intervals of 1/12 of a second. The cell at the left did not move during the sequence and it provides a reference point.

of debris or to the surface of the microscope slide⁷. These bacteria rotate rapidly. The rotation could reflect the motion of the tethered flagellum or it could result because the remaining untethered flagella cause the cell to move. To examine this motion in greater detail, we used 'polyhook' mutants of *E. coli*¹⁰, which carry two mutations, one in the *hag* gene which eliminates the formation of the flagellar filament and another in the *flaE* gene which causes the loss of a function necessary to terminate the assembly of the hook structure. These cells make continuous polyhooks which can be 1–2 μ m long and they are nonmotile. However, when dilute anti-polyhook antibody is added to a suspension of cells, they form clumps and begin to rotate rapidly. Several situations have been observed: the bacteria seem to be trapped or attached to the slide; the bacteria are bound to each other in pairs and counter-rotate or one rotates while the other is stationary; large groups of bacteria are cross linked and rotate or move with a jerking type of motion. Ten to twenty per cent of the bacteria in a microscope field can be found to be rotating. Figures 1 and 2 illustrate the first two situations. In Fig. 1, the cell seems to be attached to the surface of the microscope slide. It rotates 360° in a counterclockwise direction around an axis through one end of the cell body. The speed of rotation of an individual cell varies from two to nine revolutions per second. The cells can be observed to modulate their spinning in three ways: (a) by continuing to spin counterclockwise; (b) by stopping and then restarting in the same direction, or (c) by changing direction and spinning clockwise. The cells generally spin

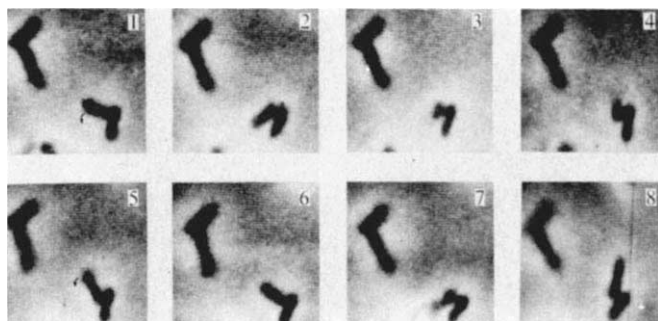


FIG. 2 The rotation of one cell bound to another. The preparation was the same as that described in the legend to Fig. 1. The cells in the upper left corner serve as reference.

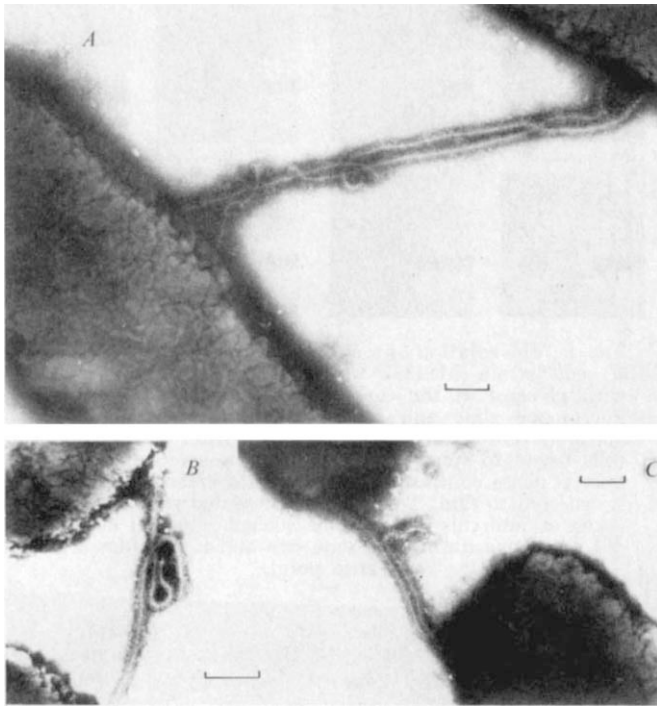


FIG. 3 Electron microscopy of cells bound together via their polyhooks. The cells were prepared as described in Fig. 1. They were placed on copper grids covered with carbon coated formover and stained with 0.5% phosphotungstic acid. The polyhook structure generally appears to be helical with a diameter of about 16 nm (ref. 10). These, however, are straightened and thickened as a result of the antibody molecules bound to them. The line in the photograph represents 0.2 nm. A, two cells held together by antibody bound to three polyhooks, B and C, cells held together by antibody bound to polyhooks derived from each of them.

counterclockwise and occasionally stop or reverse direction for one or two turns and then resume spinning counterclockwise. Individual cells tethered in this way have been observed to spin with intermittent short pauses for more than 30 m. Figure 2 illustrates the motion observed with a pair of tethered bacteria. Again the observations are most consistently interpreted as resulting from the complete rotation of one of the cells about a point of attachment to the other cell. The attachment appears as a narrow gap between the cells (Fig. 2, frame 4 and frame 8) which could correspond to the polyhook-antibody complex.

Thus, it is clear that the cells can rotate. However, it is possible that they rotate around the point of attachment, using the antibody as a swivel rather than rotating around the point of insertion of the basal structure into the cell body. Figure 3 shows electron micrographs of the tethered cells. It is clear that the polyhook structures are coated with antibody molecules, they bind the structures together at many points. The polyhooks have lost some of their characteristic helical appearance and are straightened, suggesting that they have some structural flexibility. The multiple attachment points along the surface of the polyhook appear to stitch them together and make it seem highly unlikely that the cells could rotate around the attachment site.

While these experiments were all done using the polyhook mutant, similar experiments were possible with bacteria that had flagellar filaments. *E. coli* strain W3110 which we used carries a mutation in the *hag* gene that results in the synthesis of straight flagellar filaments lacking the characteristic helical appearance. The cells are nonmotile. Nonetheless, indirect evidence suggests that the flagella are active¹¹. To decrease the number of flagella per cell so that they would not tangle and crosslink, the bacteria were grown in minimal

medium with glucose as a carbon source^{12,13}. When dilute anti-flagellar filament antibody was added to these cells they were tethered to the glass and to each other. They began to rotate in the same manner as the polyhook mutants. Thus, even with the straight flagellar filament the cell rotation is observed.

All these observations are concerned with the rotation of the cell, and the rotation of the filament is inferred. To observe the activity of the filaments themselves, we used small latex beads 0.7 μ m in diameter (Dow), which were incubated with a 1:1,000 dilution of anti-flagellar antibody and then washed in 0.01 M Tris buffer, pH 7.8, and 0.1 M sodium chloride. They were added to suspensions of *E. coli* W3110 that had been grown in minimal medium with glucose. Beads were found attached 1–2 μ m from the bacterium and they rotated very rapidly. In the same way, beads could be attached to flagella on wild-type bacteria and these were again observed to rotate rapidly, trailing behind motile bacteria.

All these observations, taken together, can be explained best if the hook is driven in a rotary fashion, probably by a mechanism anchored to the cell body at the base of the flagellum. This results in the rotation of the flagellar filament. Furthermore, the cell has the capacity to vary the speed and direction of rotation as well as the frequency of stopping and restarting. The ability to modulate the rotation of flagella may be the basis for the mechanism of chemotaxis.

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Change in direction of flagellar rotation is the basis of the chemotactic response in *Escherichia coli*

BERG and Anderson¹ recently argued from existing evidence that bacteria swim by rotation of their helical flagella. Silverman and Simon² have now provided a clear demonstration of this. By means of antibodies specific for flagellar components, they tethered cells to microscope slides or to each other and observed rotation of the cell bodies. The cells were able to stop and to rotate in either direction. It seemed possible, as they proposed³, that cessation or reversal of flagellar rotation might be involved in bacterial chemotaxis. Accordingly, we used wild-type and chemotaxis-defective mutant cells of

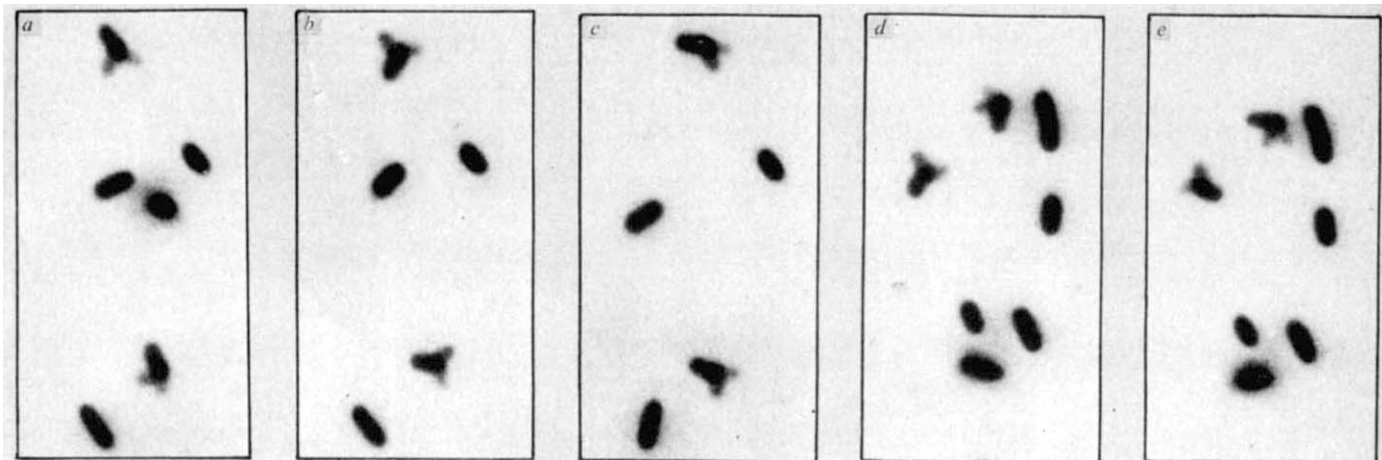


FIG. 1 Tethered cells rotating and responding to temporal chemical gradients. Rotating cells were adhered to glass slides by antibody-mediated fixation of a single flagellar filament. Bacteria were grown in glucose to reduce the number of flagella per cell^{2,9,10}. (About 30% of cells had flagella with an average number of 1.5 per cell compound with 4.3 for glycerol-grown cells as counted by electron microscopy.) This was necessary for reproducible results, presumably because additional flagella could tangle and crosslink², or cause additional torque on the cells. Bacteria, grown in liquid glucose minimal medium⁴ to logarithmic phase, were washed twice by centrifugation in chemotaxis medium (10 mM potassium phosphate, 0.1 mM potassium EDTA, pH 7.0), and resuspended in the same. A sample of bacterial suspension was mixed with 0.003 volumes of anti-filament antibody in chemotaxis medium (an amount just sufficient to produce rotation of cells), and then immediately placed on a glass microscope slide. After 2 min untethered cells were rinsed off with chemotaxis medium to improve the dark-field image of rotating cells. Addition of antibody eliminated the small proportion of free swimming cells; furthermore, less than 5% as many rotating cells were found in the absence of antibody as in its presence, and these readily washed away. A 5 μ l sample of chemotactic agent in chemotaxis medium was then added to the approximately 50 μ l drop remaining over the tethered cells, using Drummond microcapillary pipettes. Photomicrographs were taken with three exposures, each of two-fold decreasing duration 1/20 s apart, generated by three slits of decreasing width in a rotating disk in the light beam. Negative prints of dark-field images are shown. Therefore, to determine directions of rotation, follow cell bodies from darkest, to intermediate, to most faint exposure. The same field of wild type (AW405) is shown with (a) no addition, (b) addition of 0.15 M L-leucine, and (c) subsequent addition of 0.1 mM L-serine. The same field of the tumbling mutant AW620 is shown with (d) no addition, and (e) addition of 0.1 mM L-serine. (Approximate magnification is $\times 2,600$.)

Escherichia coli tethered to microscope slides in a manner similar to that of Silverman and Simon², and stimulated them by sudden increases of chemotactic agents. We found that changes in the direction of flagellar rotation indeed constitute the basis of chemotaxis: addition of attractants causes counter clockwise (CCW) rotation, whereas repellents cause clockwise (CW) rotation.

Historically bacterial chemotaxis has been identified³ and measured⁴ using an assay in which bacteria swim into a capillary tube containing attractant. More recently, chemotaxis has been studied by analysing the frequency of abrupt changes in direction of swimming (tumbles) of individual cells of *E. coli*^{5,6} and *Salmonella typhimurium*^{7,8} when they are presented with chemical gradients. Each tumble results in a new randomly chosen swimming direction⁵ such that variation of the frequency of tumbles causes a net migration of cells during a spatial chemotactic response. In this report we conclude that tumbles are caused by CW flagellar rotation whereas smooth swimming is caused by CCW rotation.

In the absence of added attractants or repellents, rotation of tethered wild-type cells as viewed from above is biased to

the CCW direction² (Fig. 1a and Table 1, strains AW607, AW405 and AW574). CCW rotation of the cell body corresponds to CCW rotation of the flagellum if viewed along the helix axis toward the point of insertion in the cell.

Changes in the direction of flagellar rotation are shown to be involved in chemotaxis by the following evidence. (1) Repellent additions (L-leucine) cause CW rotation of wild-type cells (Fig. 1b and Table 1). Note that mutants unable to respond to L-leucine in conventional tests for chemotaxis (AW608 and AW518) show no significant change. (2) Attractant additions (L-serine or L-aspartate) cause CCW rotation of wild type cells, and can even reverse CW rotation initiated by previous L-leucine addition (Table 1 and Fig. 1a, b and c). However, mutants with defective responses to L-serine or L-aspartate (AW518 and AW539 respectively)¹¹ show no response to a corresponding chemotactic stimulus (Table 1). These mutants respond normally to other chemicals (data not shown). (3) The generally non-chemotactic mutants (unable to respond to any known chemicals)^{12,13}, *cheA593*, *cheB590*, and *cheC497*, rotate only in the CCW direction, even following stimulation by L-leucine (Table 1). (4) Mutants which tumble incessantly when suspended in chemotaxis medium (for example, AW620), almost always rotate CW (Fig. 1d and Table 1). This tumbling mutant is capable of a subnormal but significant response to L-serine in a capillary assay (data not shown), and tethered cells reverse to predominantly CCW rotation when stimulated with L-serine (Fig. 1e and Table 1). (5) By adding chemotaxis medium to a drop containing tethered cells (AW405), we found that a forty-fold dilution of L-serine (from 20 μ M) reversed the cells from CCW to predominantly CW rotation for 1–2 min. A similar dilution from 20 mM L-leucine immediately reversed their previous L-leucine-stimulated CW rotation. Thus decreasing temporal gradients have the opposite effect on rotation to that of increasing gradients.

The following experiments (data not shown) were performed to substantiate the above evidence. (1) Chemotactically inert agents (chemotaxis medium, 20 mM glycerol, 20 mM D-gluconate or 20 mM DL-lactate)^{11,14} had no effect on the direction of rotation. (2) Other attractants (1 mM D-glucose, 20 mM glycine or 20 mM L-methionine)^{11,14} also cause CCW rotation. (3) Other repellents (20 mM L-isoleucine, 20 mM L-phenylalanine or 0.4 mM potassium salicylate^{14a} caused predominantly CW rotation. (4) Non-metabolisable attractants (20 mM α -aminoisobutyrate or 2 mM DL- α -methylaspartate)¹¹ elicited CCW rotation. Hence, availability of energy or metabolism of chemotactic agents is neither involved in chemotaxis^{11,15} nor in the rotational changes we observe. (5) The effects with both attractants (even the non-metabolisable analogues) and repellents were observed to decay to the original state of rotation at times ranging from one-half to several minutes after the stimulus. Thus the effects are due to temporal stimulation and not simply

TABLE 1 Rotation of tethered wild-type and taxis-defective cells before and after chemotactic stimulation

Line No.	Strains* Parent Mutant	Pertinent taxis properties	Stimulus, final concentration†	No. of cells timed	% of time in CCW rotation‡	Fisher <i>t</i> test of means Line No. compared with:	<i>P</i>
1	AW607 §	Wild type	None	10	88.2 ± 15.6		
2			30 mM L-leucine	11	0.2 ± 0.6	1	<0.001
3	AW608 §	Leucine	None	7	94.6 ± 6.5	1	0.3
4		taxis mutant	30 mM L-leucine	8	93.2 ± 8.9	3	0.7
5	AW405 ¹²	Wild type	None	30	73.0 ± 20.0		
6			3 mM L-leucine	11	20.6 ± 14.8	5	<0.001
7			30 mM L-leucine	9	3.8 ± 5.7	5	<0.001
8			20 μM L-serine	8	100.0 ± 0.0	5	<0.001
9			2 μM L-aspartate	17	100.0 ± 0.0	5	<0.001
10	AW518 ¹¹	Deficient in serine and leucine taxis §	None	14	72.6 ± 29.4	5	>0.9
11			3 mM L-leucine	8	65.6 ± 26.2	10	0.6
12			20 μM L-serine	7	78.3 ± 8.8	10	0.6
13	AW539 ¹¹	Defective in aspartate taxis	None	11	87.9 ± 9.4	5	0.03
14			2 μM L-aspartate	9	85.3 ± 11.2	13	0.6
15	<i>cheA593</i> ¹³	Generally non-chemotactic	None	18	100.0 ± 0.0	5	<0.001
16			30 mM L-leucine	14	100.0 ± 0.0		
17	<i>cheB590</i> ¹³	Generally non-chemotactic	None	29	100.0 ± 0.0	5	<0.001
18			30 mM L-leucine	8	100.0 ± 0.0		
19	<i>cheC497</i> ¹³	Generally non-chemotactic	None	10	100.0 ± 0.0	5	<0.001
20			30 mM L-leucine	6	100.0 ± 0.0		
21	AW574 ¶	Wild type	None	23	88.3 ± 9.9		
22			20 μM L-serine	11	100.0 ± 0.0	21	<0.001
23	AW620	Tumbling motility	None	19	2.3 ± 3.6	21	<0.001
24			20 μM L-serine	10	100.0 ± 0.0	23	<0.001

Cells were grown and prepared for observation as described in Fig. 1, except that 'untethered' cells were not rinsed from the slide. Rinsing is recommended when increased reproducibility is required. Occasionally, a cell would appear to become stuck, or to change its centre of rotation during timing. Data from such cells were not included.

* All strains are derivatives of *E. coli* K12.

† Five or 10 μl of stimulant in chemotaxis medium were added to 20 μl drops covering tethered cells by using Drummond microcapillary pipettes.

‡ Each cell was observed for 30 s beginning immediately after stimulation. Times were determined manually with one stop clock for CW and another for CCW rotation. The infrequently observed short periods of no rotation were not included in the calculations.

§ See ref. 14a

¶ A *gal*⁺, *su*⁻ derivative of AW405.

to presence of the chemical. (6) Rotational responses of several strains before and after stimulation were confirmed by high speed microcinematography. (7) Tethered cells of the polyhook mutant² showed rotational responses to stimulation identical to wild-type cells. (8) Tethered cells of a paralysed mutant (flagellated but non-motile)¹³ did not rotate even in response to attractants or repellents.

From the above results we conclude that changes in direction of flagellar rotation are the basis of both positive (toward attractants) and negative (away from repellents) chemotactic responses.

As mentioned above, chemotaxis occurs by means of variation of the tumbling frequency. Increasing attractant concentrations (whether encountered as temporal stimuli, or while swimming in spatial gradients) cause cells to tumble less frequently⁵⁻⁸, while increasing repellent concentrations cause more tumbling⁸. On the other hand, decreasing concentrations cause the opposite effect (more tumbling for attractants and less tumbling for repellents)^{7,8}. What, then, is the relationship between direction of flagellar rotation and tumbles?

The generally non-chemotactic mutants swim smoothly with virtually no tumbles¹⁰ and, when tethered, never rotate in the CW direction (Table 1). On the other hand, mutants which tumble continually rotate their filaments in the CW direction most of the time (Table 1). Furthermore, these mutants swim smoothly and rotate their filaments predominantly in the CCW direction only when stimulated by attractants. From these observations, as well as the effects of attractants and repellents on rotation of wild-type cells (Fig. 1a, b and c, and Table 1), we conclude that tumbling occurs as a result of CW rotation of flagella.

Peritrichously flagellated bacteria swim by coordination of several helical filaments in a bundle behind the cell¹⁴. Bacteria with predominantly smooth swimming (wild-type, generally non-chemotactic mutants, and responsive strains

when attractant was added) all rotate their filaments predominantly in the CCW direction. Conversely, bacteria induced to tumble rotate their filaments CW. We therefore conclude that to obtain the thrust required for smooth swimming from CCW rotating filaments in a bundle, the helical sense of the filaments of *E. coli* must be left-handed. It is significant that the helix of filaments of *Proteus vulgaris*, another enteric Gram negative peritrichously flagellated bacterium, is also left-handed^{17,18}.

Although it is possible that cessation of rotation or differential rates of CCW rotation of filaments could cause a tumble, we believe that CW rotation is the most likely, cause, because tethered cells of tumbling mutants rarely stop rotating and seldom rotate CCW; whereas, when not tethered, they tumble very frequently. The same can also be said for cells responding to repellents. Thus cessation of rotation does not correlate with tumbling. Although we have no evidence against involvement of differential rates of flagellar rotation in tumbling, it is not necessary to invoke them in the mechanism of chemotaxis. Clockwise rotation of one or more left-handed helical filaments can lead to tangling or, more probably, dissociation of flagellar bundles, and thereby generate tumbles.

Several interesting questions arise now that it is known that flagella can rotate in either direction² and that this ability is basic to chemotactic responses. As proposed by Berg and Anderson¹, the morphology of the flagellar basal complex and its association with the cell envelope^{19,20} is suggestive of a cylindrically symmetrical rotary 'motor'. Are there separate motors for CCW and CW rotation, or does a single motor reverse? Do the motors rotate by step-wise molecular interactions, or as 'turbines' driven by ion fluxes? How do chemotactic stimuli, acting through chemoreceptors¹⁵, control the direction of rotation? Do chemoreceptors communicate only with a nearby flagellum, or simultaneously with all flagella?

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Dynamic properties of bacterial flagellar motors

TETHERED bacteria¹ rotate at the angular velocity at which the torque generated by the flagellar motor² is balanced by the torque due to the viscous drag. In general, $M = b\eta\Omega$, where M is the torque, η is the viscosity, Ω is the angular velocity, and b is a coefficient which depends on the size and the shape of the cell, the position of the axis of rotation, and the distance between the cell and the wall. For a sphere of radius a (not too close to the wall) $M = 8\pi\eta a^3\Omega$ (ref. 3). Viscous forces are so large in comparison with inertial forces⁴ that Ω will change with M virtually instantaneously; any discontinuities in the one will be evident in the other. Consider a cell of radius a and uniform density ρ rotating at an angular velocity Ω_0 ; if its motor is suddenly disengaged, Ω will decay exponentially to 0 with a time constant $\rho a^2/15\eta$, and the cell will stop in $\Omega_0 \rho a^2/15\eta$ radians. For *Escherichia coli* this is less than a millionth of a revolution. The cell also is subject to rotational diffusion, but this will be evident only if the coupling between the flagellum and the body of the cell is fluid. The root-mean-square deviation in the angular position is $(2Dt)^{1/2}$, where D is the rotational diffusion

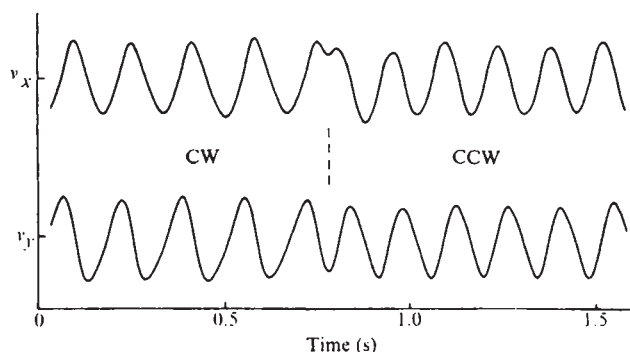


FIG. 1 The rotation of a polyhook mutant bound to the top window of the tracking chamber. The cell rotated clockwise (CW) at 6.2 r.p.s. and then counterclockwise (CCW) at 7.2 r.p.s. (The x velocity lags the y velocity for CW rotation but leads it for CCW rotation; the phase shift is 90°.) *E. coli* strain MS1381 was grown on minimal medium containing glycerol as described by Silverman and Simon⁶, washed twice with a solution containing 10^{-2} M potassium phosphate (pH 7.0) and 10^{-4} M ethylenediamine tetraacetate, and suspended in a similar medium containing 0.067 M sodium chloride (10^8 cells ml^{-1}). Antipolyhook antibody was added to a dilution of 1:20,000, and the window was immersed in this mixture. It was removed 20 min later and inverted onto the top of a tracking chamber (filled with the suspension medium). The cells were tracked at 32°C. The microscope readout voltages (ref. 5, Figs 1 and 5) were amplified, differentiated, and displayed on a strip-chart recorder. The frequency response of this system was checked with sine waves of amplitude 0.05 and frequency 20 times that of the signals which generated the recording shown in the figure; the test signals were readily detectable. Note that the signals generated by the rotation of the cell are completely smooth.

constant and t is the time. For a cell which can rotate freely, $D = kT/b\eta$, where k is Boltzmann's constant and T is the absolute temperature.

The rotation of *E. coli* can be monitored with the tracking microscope⁵, which follows the point on the surface of the cell closest to the centre of the laboratory reference frame. If the cell rotates counterclockwise ($\Omega > 0$), the transducer traces out a counterclockwise path. If the cell is seen from the side and is tethered near one end, the x and y velocities of the tracker vary with time roughly as $\Omega \sin \Omega t$ and $-\Omega \cos \Omega t$ (Figs 1 and 2). The exact shape of the waveform depends on the size and the shape of the cell and on the position of the rotation axis.

The motion can be remarkably smooth. If the motor makes one revolution in n discrete steps, I conclude from data of the kind shown in Fig. 1 (for cells rotating as slowly as 4 r.p.s.) that $n \gtrsim 25$. This probably rules out the mechanism envisaged earlier², in which a cycle is executed when each of three (or more) cross bridges takes nine steps. This mechanism could explain the data only if the motions of the different cross bridges were highly synchronised. If the response time of the tracking and recording system is τ , then the root-mean-square deviation in Ω due to rotational diffusion should be of order $(2D/\tau)^{1/2}$. Under the conditions of the experiment ($\tau \approx 10^{-2}$ s), the value of the deviation expected for a freely rotating object the size of *E. coli* is about 6 s⁻¹. The data are much smoother than this (Fig. 1), so the coupling between the flagellum and the body of the cell is not fluid.

Changes in the direction of rotation¹ occur smoothly, yet abruptly (Figs 1 and 2), and they occur at different points in the cycle (Fig. 2 and data obtained with the polyhook mutant, not shown). The data at hand are consistent with a model in which the reversals occur at random⁹. The angular velocities for clockwise and counterclockwise rotation are generally different (Figs 1 and 2), but this does not necessarily mean that the drive is asymmetric. When the motor reverses, the torque applied to the hook and the filament changes

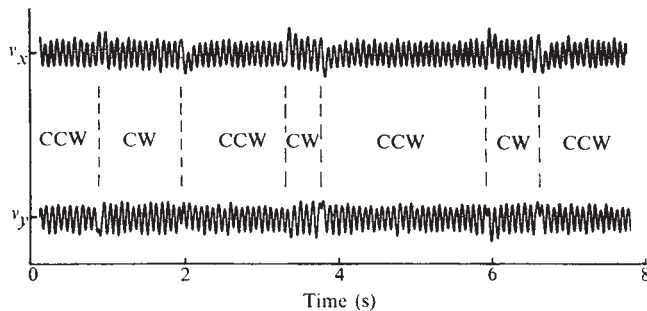


FIG. 2 The rotation of a wild type cell bound to the top window of the tracking chamber. In the interval shown the cell changed direction six times; it ran 71 cycles CCW at 13.2 ± 0.2 r.p.s. and 28 cycles CW at 12.4 ± 0.8 r.p.s. The reversals occurred at different points in the cycle. The cell was followed for a total of 6 min; the interval shown is typical of the entire record, except that the cell stopped occasionally for about 0.2 s and then started again in the same direction. *E. coli* strain AW405 (ref. 7) was grown, washed, and linked to the window by the methods of Larsen *et al.*⁸ Other conditions were as described in Fig. 1.

sign; if either one changes its shape, the orientation of the cell (and thus the load) may be different. Note that the amplitudes of the signals (Fig. 1 or Fig. 2) are larger when the frequencies are lower.

Cells which rotate with larger radii of gyration tend to rotate more slowly, which implies that the motors slow down with increasing load. This was confirmed quantitatively in experiments with the polyhook mutant in which Ω was measured as a function of η (Table 1). When the motors run more slowly, they deliver somewhat more torque but less power. Since the torque is approximately constant, a cell tethered by an inert structure, such as a pilus (the point of attachment assumed fluid), and driven by a free helical filament (rotating rapidly on the opposite side of the cell) should rotate at about the same rate as a cell tethered by the flagellum itself (the point of attachment assumed rigid). I have seen this with wild type cells: in the absence of antibody, as many as 90% spin the wrong way (at the usual rates but predominantly clockwise on the slide, counter-clockwise under the coverslip). As Silverman and Simon¹ note in the accompanying report, a distinction between rotation and other means of propulsion cannot be made with tethered cells unless they are non-motile when free.

Cells rotate perfectly well at high osmotic pressures, even when visibly plasmolysed (experiments with the polyhook mutant in 0.25 and 0.50 M sucrose). When the osmotic strength is increased, some cells slow down, but most continue

to rotate rapidly (Ω in the range expected from changes in η). At higher osmotic strengths (0.75 M sucrose) most cells stop. These effects are reversible. The results are consistent with those obtained by Vaituzis and Doetsch^{10,11}, who found that flagellated spheroplasts swim in a medium isotonic to the cytoplasm provided that they retain remnants of the cell wall. Severe plasmolysis may cause reversible disruption of flagellar basal structures¹².

A model for a flagellar motor is presented in Fig. 3. The motor operates with two rings (the M and S rings), since only two have been found in Gram positive bacteria¹³. Four have been identified in Gram negative bacteria¹³, but the outer pair probably serve as part of a bushing required for passage of the rod through the multilayered wall. The M ring has been shown to be associated with the cytoplasmic membrane in both Gram positive and Gram negative cells¹⁴, but specific attachments of the S ring to known cell envelope structures have not been found. The motor must be attached to the wall somewhere, or else the torque which it can generate cannot be applied. If the rings are pulled away from the wall¹² or if the wall is dissolved away¹¹, the rings may continue to rotate relative to one another, but they will not drive the cell.

Lubrication does not seem to be a problem, the reason being that everything is very small. I have computed the

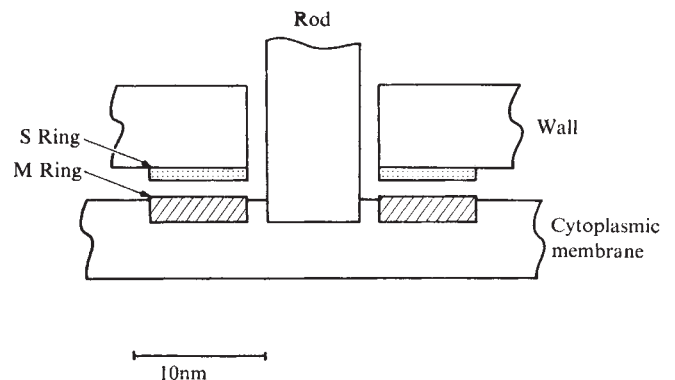


FIG. 3 A schematic representation of a flagellar motor. The rod is connected to a filament by a proximal hook (not shown). The torque is generated between the M ring and the S ring. The M ring, which is mounted rigidly on the rod (connections not shown), rotates freely in the cytoplasmic membrane. The S ring is mounted on the wall. The radius of the rod is 3.5 nm and the inner and outer radii of the rings are 5 nm and 11.2 nm, respectively¹³. (The wall thickness and the space between the M and S rings are not to scale.)

TABLE 1 Changes in angular velocity, torque and power dissipation resulting from changes in viscosity

Relative change in viscosity	2.42*	2.31†
Relative change in angular velocity	0.50 ± 0.08	0.51 ± 0.09
Relative change in external torque	1.21 ± 0.19	1.18 ± 0.21
Relative change in power dissipation	0.61 ± 0.22	0.60 ± 0.19

Cells were tethered to microscope coverslips by the method described in the legend of Fig. 1 and examined manually at 32°C. Cells rotating at uniform rates (0.3–3 r.p.s.) were clocked for about 1 min, the medium was replaced with one containing more (or less) methylcellulose (Dow Methocel 90 HG), and the measurements were repeated. Since the cells had different shapes and sizes and were tethered in different ways, the relative changes in Ω (and hence in the torque $b\eta\Omega$ and in the power dissipation $b\eta\Omega^2$) were computed for each cell separately. The data were obtained from twenty-one paired measurements on ten cells from four cultures. The standard deviations shown in the table were computed by weighting each pair of measurements equally. The viscosities were determined at 32°C with a Cannon-Ubbelohde viscometer.

* 0.776 to 1.88 cP.

† 1.88 to 4.35 cP.

torque due to the viscous shear in the space between the rod and the wall and in the space between the M and the S rings³ and approximated the torque due to the rotation of the M ring in the membrane by half the torque on a sphere of similar radius. If the passage through the wall is 20 nm long, the gap between the M and the S rings is 0.5 nm thick, and the viscosity is 1 P (see Fig. 3 for the other dimensions), the internal drag is only 3×10^{-4} times as large as the drag on the body of the cell.

The motor must bear both axial and radial loads, since the rod in a peritrichously flagellated bacterium rarely lies along the direction of motion. The forces are of order $6\pi\eta av$, where a is the radius of the cell (approximated as a sphere) and v is its speed; that is, of order 10^{-8} dynes. When an intact cell swims in a dilute solution, the axial load is more than balanced by osmotic pressure, which forces the M and S rings out against the wall (a force of order 10^{-5} dynes); when a spheroplast swims in a medium isotonic to its cytoplasm, the axial load is not balanced in this manner. As noted above, the coupling between the flagellum and the body of the cell

is not fluid, so the axial and the radial loads can be borne at the point of contact (between the M and the S rings).

How is the torque generated? Larsen *et al.*¹⁵ have found that an intermediate in oxidative phosphorylation (but not ATP) is required for motility in *E. coli*. This suggests a mechanism in which the movement of one molecule down an electrochemical gradient (through the membrane) causes another molecule to exert a force on the S ring in a direction parallel to its face but normal to its radius. A force in the opposite direction could result from a change in the coupling between these molecules or from the action of an independent set. Since the power dissipation for an *E. coli* spinning 10 r.p.s. is of order 10^{-9} erg s⁻¹ and the energy which can be gained from the transit of one molecule through the membrane is of order 10^{-13} erg, 10^3 such events could drive the cell through one cycle. The individual steps would be too small to be seen in the present experiments.

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Presence of HCN in *Chlorella vulgaris* and its possible role in controlling the reduction of nitrate

NITRATE reductase of *Chlorella vulgaris* is present in crude extracts mainly in an inactive form¹⁻³ which may be converted to an active form by the addition of an artificial oxidant such as ferricyanide⁴. In crude extracts from which low-molecular weight substances have been removed by gel filtration, added NADH (or NADPH) causes a rapid conversion of the active form of nitrate reductase to the inactive form⁵. Losada and coworkers have also observed an inactivation of the nitrate reductase from *Chlorella fusca*⁶ and *Chlamydomonas reinhardtii*⁷ by added NADH. A low-molecular weight fraction separated from crude extracts of *Chlorella vulgaris* also causes an inactivation of nitrate reductase when added back to the extract which had been treated with ferricyanide and passed through a Sephadex column to remove excess reagent⁵. In all cases the enzyme can be reactivated by the addition of the oxidant, ferricyanide⁴⁻⁸. On purification of the enzyme, Solomonson⁹

found that two substances must be present simultaneously to cause the reversible inactivation: (1) a reducing substance, preferably NADH, and (2) a substance that behaves like HCN. The molar concentration of HCN needed to effect a rapid and reversible inactivation of the enzyme was of about the same magnitude as the enzyme concentration. Thus, activated, suitably purified nitrate reductase with added excess NADH, provides an exceedingly sensitive, though non-specific test for HCN, as shown in Fig. 1.

Although this bioassay could not be applied directly to extracts, it could easily be used to show that distillates prepared from *Chlorella* cells, or from extracts of *Chlorella* cells, contained something which behaved like HCN. The concentration of the unknown substance could be increased by redistillation until it came within the sensitivity range of specific chemical tests, which showed that the substance was, indeed, HCN. After many preliminary experiments, conditions were found for obtaining sufficiently high HCN concentrations in the first distillate to come within the range of a chemical test as well as of the bioassay. The results of a series of such experiments, given in Table 1, show that the amount of HCN which could be obtained from freshly collected *Chlorella* cells was somewhat greater than the amount of nitrate reductase present in the cells. Thus, the quantity of HCN found can account for the fact

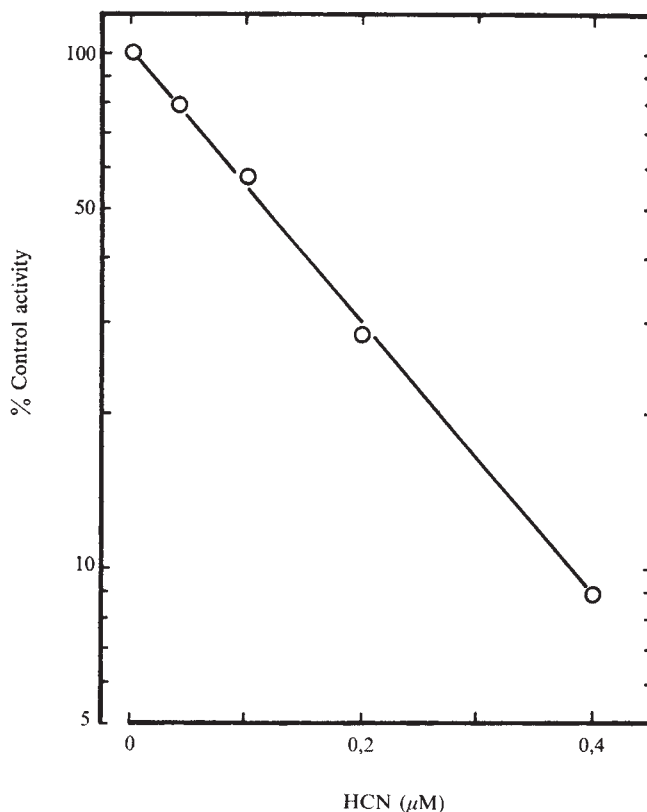


Fig. 1 A calibration curve for the bioassay for HCN. The percentage inactivation of nitrate reductase is plotted on a log scale against the initial HCN concentration. Incubation mixtures containing 0.05 M Na,K phosphate buffer (pH 7.6), 0.2 mM NADH, and the indicated concentrations of HCN were brought to 20° C. The inactivation was initiated by addition of activated enzyme to a concentration of 0.86 units ml⁻¹. After 6 min, aliquots were added to the enzyme assay mixture and activity was assayed as previously described¹⁻⁹. A control inactivation mixture without cyanide, gave 95% of the initial activity. This was taken as 100% for calculation of the percentage inactivation. It is essential that all HCN be removed from the enzyme preparation, so that NADH alone gives a negligible inactivation. The preparation of the enzyme is described elsewhere⁹. The sensitivity of this bioassay can be increased by decreasing the concentration of enzyme.

TABLE 1 HCN from *Chlorella* cells

Cells (ml)	HCN (nmol per ml cells)	
	Bioassay	Chemical assay
23.7	1.22	1.38
21.3	1.27	1.62
28.0	1.26	1.39
27.5	2.05	1.75
(Mean)	1.45	1.53

Chlorella vulgaris was grown under the conditions used for the preparation of nitrate reductase^{1,5,9,23}. HCN was separated by simple distillation. In the first experiment, 23.7 ml freshly collected, packed cells were washed with 500 ml redistilled water and suspended in a distillation flask in enough water to give 0.5 ml cells per ml suspension. An equal volume of 1 N H₂SO₄ was added, and distillation was begun immediately. The condenser was well cooled, and the receiving flask was held deep in an ice bath. Several fractions of distillate were collected, until no further active material was obtained. Over 90% of the total yield of HCN was obtained in the first 17 ml of distillate. Other experiments were done with minor variations. Control experiments with known HCN showed that there was negligible loss of HCN under these conditions. Another control experiment with tracer H¹⁴CN showed that the ¹⁴C distributed itself with the chemical assay and the bioassay. Addition of alkali to the receiving flask was unnecessary and was avoided because other substances present in the distillate reacted with the HCN at alkaline pH.

The cells used for these experiments generally gave about 1.4 units of activated enzyme per ml of sonicated extract, corresponding to about 200 μ l of cells. This is a yield of 7.0 units per ml cells. From the turnover number², we estimate that one unit of enzyme is about 0.09 nmol, which gives 0.63 nmol enzyme per ml cells, compared with 1.2 to 2.0 nmol HCN. The estimate of the amount of enzyme present is not very accurate, since we have no reliable measure of the losses sustained during the extraction procedure.

The chemical assay was carried out according to Guilbault and Kramer¹¹, without addition of isonitrosobenzoylacetone. Since cyanide acts catalytically in this test, the rate of colour formation was measured at 23°C. HCN (0.1 nmol) added to the test system gave $\Delta A_{560\text{nm}} = 0.0365$ in 5 min ($d = 1$ cm).

that the nitrate reductase present is largely in the inactive form.

Our current procedure for purifying nitrate reductase began with a protamine sulphate fractionation. This was carried out on a small scale, to show that the protamine sulphate precipitate, containing the enzyme largely in inactive form, yielded an amount of HCN which was approximately equivalent to the amount of inactive enzyme present. Thus, 32.5 ml extract (frozen, thawed, and cleared by centrifugation), which is approximately equivalent to 6.5 ml of cells, contained 46.3 units of inactive nitrate reductase (measured after activation of a separate aliquot by ferri cyanide) and 1.8 units active nitrate reductase. To this extract, 1 ml of 2% protamine sulphate (pH 7.0) was added, and the precipitate obtained was removed by centrifugation. Another 1 ml, and then 0.5 ml of protamine sulphate were added, the precipitate each time being removed by centrifugation. From the supernatant, essentially all of the enzyme was precipitated by further addition of 2 ml 2% protamine sulphate. This precipitate was washed twice with 0.02 M Tris buffer (pH 8.1), suspended in 4 ml of 0.1 M phosphate (pH 7.2) containing 0.1 mM dithioerythritol and 0.1 mM EDTA, and brought to 10 ml with redistilled water in a 100 ml distillation flask. An equal volume of 1 N H₂SO₄ was added, and distillation was carried out as described in the legend for Table 1. Special care was required because of foaming.

The first fraction of 1.0 ml and a second fraction of 0.9 ml distillate were collected. The bioassay gave 2.86 and 0.45 nmol HCN in the first and second distillates, respectively; the chemical assay gave 2.3 and about 0.36 nmol. The total of 3.3 nmol by bioassay compared with 2.7 nmol by chemical assay. The 46 units of inactive enzyme originally present are equivalent to about 4.2 nmol enzyme, some of

which must have been lost or activated during the extensive manipulation. The value of 4 thus compares quite well with the value of 3 nmol HCN recovered by distillation. In a control experiment, an equivalent amount of protamine sulphate gave no detectable HCN.

In another experiment, the final supernatant from the protamine sulphate precipitation was distilled after acidification. Here, the HCN recovered amounted to 3.7 nmol HCN per ml *Chlorella* used to make the extract. Adding the 0.6 nmol HCN removed with the inactive enzyme, this gives a total of 4.3 nmol HCN per ml cells, recovered from the extracts of *Chlorella*, compared with about 1.5 nmol per ml as the average recovered from the intact cells (Table 1). This is presented as evidence that cyanide formation occurs in the extract. In these experiments, distillates were examined by the chemical method of Epstein¹⁰ as well as by the bioassay and by the chemical method of Guilbault and Kramer¹¹. All methods gave comparable results. By the use of similar procedures, HCN could also be obtained from spinach leaves and from the blue-green alga, *Plectonema boryanum* (Gewitz and Völker, unpublished). These and further experiments will be described in detail elsewhere.

It has long been a puzzle that cyanide-utilising enzyme systems are present in cells not known to make cyanide¹²⁻¹⁴. Thus, Conn and his associates found in 1963¹⁵, that added H¹⁴CN is converted to the β -carboxamide group of asparagine by seedlings of some so-called non-cyanogenic plants and also of some cyanogenic plants. Tschiersch¹⁴ made similar independent observations. Fowden and Bell¹⁶ showed that *Chlorella pyrenoidosa* converted H¹⁴CN to β -cyanoalanine, and thence to asparagine or to the peptide γ -glutamyl- β -cyanoalanine. The literature on HCN metabolism has been reviewed by Blumenthal *et al.*¹⁷, who noted particularly that enzymes for the metabolic conversions of HCN are widely distributed in bacteria, fungi, algae and higher plants. Most recently, Taylorson and Hendricks¹⁸ have described a stimulatory effect of HCN on seed germination, and ascribed it to the specific synthesis of asparagine, which may be rate limiting in protein synthesis.

We propose that HCN may play an important part in a delicately balanced system which controls the activity of nitrate reductase. The nature of the chemical interaction between cyanide and nitrate reductase has not been firmly established (see ref. 9 for discussion). The fact that cyanide inhibits the enzyme has long been known¹⁹. Relimpio *et al.*²⁰ and Solomonson and Vennesland² observed that the inhibition by cyanide was enhanced by NADH. This cyanide inhibition was slowly reversed by nitrate² and rapidly reversed by added ferri cyanide⁵. The reversal of cyanide inhibition by nitrate had at least a superficial similarity to the activation of nitrate reductase in fresh extracts of *Chlorella vulgaris*². Maranville²¹ reported an increase in nitrate reductase activity in extracts from sorghum when nickel salts were added to the extraction buffer. He postulated that the nickel acts by binding cyanide released by the hydrolysis of the cyanogenic glycoside, dhurrin. We also noted a similar increase in activity when NiSO₄ was added to fresh extracts of *Chlorella vulgaris*² but the stimulation of activity by nickel was small by comparison with activation achieved by other methods.

We note that when a plant is called non-cyanogenic, this only means that the amount of HCN present is less than the amount which can be detected by the test used, or that no cyanogenic glycosides have been found in the material^{14,22}. Our experiments show that *Chlorella vulgaris*, previously considered non-cyanogenic, actually contains HCN in bound form, some of it complexed to an inactive form of the enzyme, nitrate reductase. Extracts of these cells contain considerably more HCN, which must be formed from an as yet unidentified precursor.

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Latex spheres as markers for studies of cell surface receptors by scanning electron microscopy

THE distribution of antigens and carbohydrate residues on the surfaces of cells, notably erythrocytes and lymphocytes, can be determined by binding antibodies or lectins to such macromolecules as ferritin^{1,2}, haemocyanin^{3,4} or peroxidase^{2,5}, which serve as markers for transmission electron microscopy. Improved techniques in high resolution scanning electron microscopy (SEM), however, make it possible to determine the topographical distribution of molecular receptors on the surfaces of cells and tissues by similar histochemical techniques involving markers resolvable by SEM. LoBuglio *et al.*⁶ used commercial polystyrene latex particles, 2,300 Å in diameter, as immunological markers for SEM work. But applications of this reagent are limited because the hydrophobic surface of the particles makes them stick non-specifically to many surfaces and molecules.

We have developed a more useful technique, and report here: (1) the design and synthesis of latex spheres equal to or less than 1,300 Å in diameter; (2) the covalent bonding of antibodies and other molecules to these spheres, and (3) the application of antibody-tagged particles as specific cell surface markers for SEM.

The rationale for the design of polymeric latex is based on the need for small, stable particles which are biocompatible—that is, they do not interact non-specifically with cells or other biological components—and contain functional groups to which specific proteins and other biochemical molecules can be bonded covalently. A crosslinked copolymer of hydroxyethylmethacrylate (HEMA), methacrylic acid and methylmethacrylate has these characteristics: poly HEMA has blood compatible properties and is used in the manufacture of contact lenses⁷. The hydroxyl groups can be activated by cyanogen bromide^{8,9} for covalent bonding of proteins to the polymeric latex. Methacrylic acid residues which impart a negative charge onto the particles prevent non-specific binding to cell surfaces and provide carboxyl groups to which various biochemical molecules can be bonded by the carbodiimide method¹⁰. Cross linking of the polymeric matrix is essential to maintain the stability and size of the particles in both aqueous solutions and organic solvents commonly used in the fixation and dehydration of biological specimens for electron microscopy.

Latex spheres were synthesised by aqueous emulsion copolymerisation of methylmethacrylate (MMA), HEMA and methacrylic acid (MAA) in the presence of the emulsifier sodium dodecylsulphate, the free radical initiator ammonium persulphate, and the cross linking agent trimethylol propane trimethacrylate. Polymerisation was carried out in closed tumbling containers, under argon at $98^\circ \pm 2^\circ$ C, for 6 h. The chemical composition of the latex was: MMA, 57%; HEMA, 30%; MAA, 10%, and crosslinker, 3%. By varying the polymerisation conditions the diameter of the spheres could be changed from 600 to 1,300 Å. The emulsifier and other impurities were removed by passage over a mixed bed ion exchange column.

Proteins and other molecules containing amino groups were bonded covalently to the latex spheres by either the cyanogen bromide⁸ or carbodiimide methods¹⁰. For use as visual markers in fluorescent microscopy or as reagents in quantitative studies, latex spheres ($20\text{--}50\text{ mg ml}^{-1}$) activated with cyanogen bromide (50 mg ml^{-1}) were tagged with the fluorescent dye, dansyl- ϵ -lysine, or ^3H -lysine (1 mM). Antibodies ($0.2\text{--}1\text{ mg ml}^{-1}$) were covalently bonded to latex spheres previously derivatised with ϵ -amino caproic acid using the coupling reagent, 1-ethyl-3-(3-dimethyl amino-propyl) carbodiimide (2 mg ml⁻¹) or spheres derivatised with diaminoheptane using glutaraldehyde (1.25%). Further details of the synthesis and coupling procedures will be described elsewhere.

The application of these immunochemical reagents as visual markers of cell surface components for light and electron microscopy was tested using the indirect or 'sandwich' technique⁵. Purified goat antibodies directed against rabbit γ -globulin (goat anti-rabbit) were coupled to the latex spheres. Unbound antibodies were removed by repeated centrifugation (35,000g, 30 min) and redispersion in phosphate-buffered saline (PBS). The immunochemical activity of the latex bound goat anti-rabbit antibody was verified by the agglutination of the latex in the presence of rabbit IgG.

Washed bovine red blood cells (RBC) sensitised with rabbit antiserum against bovine red blood cells (non-specific rabbit antiserum was used in the control) were incubated with goat anti-rabbit-latex conjugates ($1\text{--}20\text{ mg ml}^{-1}$) for 20 min at 20° C. After removal of the excess reagent by repeated centrifugation of the cells at 400g, the RBC were fixed in 1.25% glutaraldehyde and prepared for examination

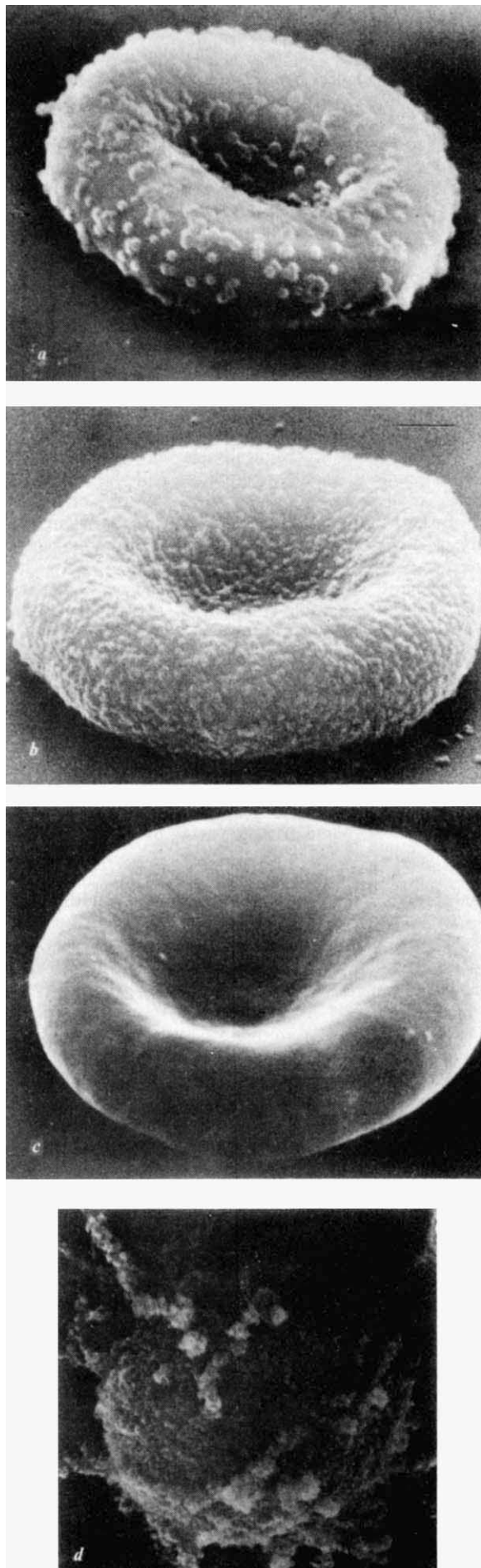


FIG. 1 Scanning electron micrograph of bovine RBC. *a*, Red blood cell labelled with rabbit anti-bovine erythrocyte antiserum followed by goat anti-rabbit antibodies covalently bonded to latex particles 1,300 Å in diameter. Only partial labelling of the cell is observed because of the low concentration of latex conjugate used. *b*, Red blood cell labelled as in (*a*) but with a higher concentration of latex particles 800 Å in diameter. (Horizontal bar represents 1 μm.) *c*, Control experiment. Red blood cell treated with non-specific rabbit antibodies followed by goat anti-rabbit antibodies conjugated to latex particles 800 Å in diameter. *d*, Mouse spleen lymphocyte labelled with rabbit anti-mouse lymphocyte serum followed by goat anti-rabbit antibodies conjugated to 800 Å latex particles. Sodium azide (5 mM) was present during labelling to prevent cap formation.

by SEM¹¹. A scanning electron micrograph of a bovine red blood cell specifically labelled with a low concentration of immunolabel particles of average diameter 1,300 Å is shown in Fig. 1*a*. A red blood cell labelled with immunolabel particles of average diameter 800 Å is shown in Fig. 1*b*. The absence of significant non-specific binding of these immunochemical reagents is illustrated in the control experiment (Fig. 1*c*). A mouse spleen lymphocyte settled onto a glass cover slip and labelled first with rabbit anti-mouse lymphocyte serum and then with goat anti-rabbit-latex conjugate (20 mg ml⁻¹) is shown in Fig. 1*d*. The latex spheres coat the surface of the cell and line the microvilli.

These reagents have several advantages for studies in the mapping of specific cell surface components. They can be easily prepared and coupled to proteins and other molecules by standard chemical procedures, they are stable indefinitely, and do not bind non-specifically to cell surfaces. They can be synthesised in various size ranges and chemical compositions to suit particular requirements. For example, smaller spheres in the size range 200–300 Å in diameter can serve as molecular markers (1) in replica and shadowing techniques for high resolution transmission electron microscopy, and (2) in double labelling experiments in conjunction with larger latex particles or with such biological markers as haemocyanin distinguishable by its cylindrical shape³. For use with fluorescent microscopy and for quantitative studies, these reagents have the advantage that by binding fluorescent dyes and radioactive molecules to the latex instead of the antibody, a high degree of tagging can be attained without adversely affecting the antibody activity for studies requiring high sensitivity. These particles when tagged with metals or other suitable elements have application in electron beam microprobe analysis and related X-ray techniques.

The application of these multipurpose reagents in studies of antigens on the surface of more complex cell and tissue specimens is being investigated. Latex particles bound to certain hormones, lectins or toxins should prove valuable in the detection and localisation of various specific receptors on cell surfaces.

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Calcium uptake by sarcoplasmic reticulum of muscle from vitamin D-deficient rabbits

RECENT rapid advances in understanding the metabolism and action of vitamin D have confirmed its primary effect on intestinal calcium transport and continue to elucidate its action on bone and kidney^{1,2}. Biochemical research has, however, largely neglected the well-known clinical observation of proximal muscle weakness in most vitamin D-deficient states³. Those studies which have been done provide only indirect evidence of a link between vitamin D and muscle. Thus, autoradiography after pharmacological doses of radioactive vitamin D has shown its localisation under the sarcolemma⁴, and when more physiological doses are given to rachitic animals, vitamin D and its metabolites may be found in significant amounts in muscle⁵. Skeletal muscle also contains a protein similar to the binding protein of kidney (but not of plasma) that binds 25-hydroxycholecalciferol⁶.

We have recently measured the uptake of calcium by the isolated sarcoplasmic reticulum (SR) of the psoas muscles of vitamin D-deficient and vitamin D-supplemented rabbits and our results provide direct evidence of a biochemical effect of vitamin D deficiency on striated muscle.

Weanling Dutch rabbits were fed a vitamin D-deficient diet (General Biochemicals, Laboratory Park, Chagrin Falls, Ohio) from the age of 4 weeks (D- rabbits). Their litter mates were given the same diet supplemented with vitamin D providing 10–13 IU vitamin D₂ d⁻¹ (D+ rabbits, 'controls'). The D- rabbits became weaker than the controls, and showed histological and biochemical evidence of rickets, (plasma calcium 7.9 ± 0.4 mg per 100 ml; control 12.8 ± 0.4 ; mean $\pm$ s.e.m.). At the end of the period on the diet (5–7 weeks), pairs of D- and D+ animals were killed and both psoas muscles were quickly excised from each animal and placed in ice-cold buffer (5 mM histidine, pH 7.0; 0.1 M KCl). Individual muscles were homogenised and an SR fraction isolated, sedimenting between 8,000 and 70,000g, by differential centrifugation, according to Martonosi, Donley and Halpin⁷, except that only one extraction in 0.6 M KCl was carried out. The final SR pellet was resuspended in 5 mM histidine, pH 7.0, containing 0.1 M KCl and 5 mM dithiothreitol. Electron microscopy showed little contamination of the vesicular material by either sarcotubular fragments or mitochondria. The rate of calcium uptake by SR was measured on the day after isolation by Millipore filtration assay (Table 1)^{8,9}. Preliminary experiments showed that calcium uptake was dependent on ATP. Its rate varied linearly with SR protein concentration (10–40 μ g protein ml⁻¹), and with time for the first 90 s of the reaction. The rate of calcium uptake over the first minute by the SR from each muscle was measured in duplicate at three dif-

TABLE 1 Rate of calcium uptake by SR isolated from the psoas muscles of D+ and D- rabbits

Pair No.	Calcium uptake (μ mole per min per mg SR protein)	
	D+	D-
1	1.94	1.42
2	2.13	1.88
3	1.88	1.68
4	2.06	1.55
5	2.19	1.65
6	1.73	1.23
7	1.74	1.25
8	1.00	0.81
Mean $\pm$ s.e.m.	1.83 ± 0.13	1.43 ± 0.12

Calcium uptake was measured in 3 ml of medium containing 20 mM histidine, pH 6.4, 100 mM KCl, 5 mM Mg²⁺, 5 mM oxalate, 5 mM ATP, 100 μ M⁴⁵ CaCl₂ and 10–40 μ g SR protein ml⁻¹ at 25 °C. The reaction was started by the adding of ⁴⁵CaCl₂ and stopped, after 60 s, by passing an 0.1 ml sample, diluted with 0.5 ml of 5 mM histidine, pH 7.0, 0.1 M KCl buffer, through an HA Millipore filter (pore size 0.45 μ m). The rate of calcium uptake by the SR was calculated from the ratio of the amounts of ⁴⁵Ca, determined by liquid scintillation counting, retained on the filter and in the filtrate. Blank values were obtained in the absence of SR protein. The values for each rabbit are the average of twelve separate determinations (see text).

ferent protein concentrations. As the mean value obtained from the psoas muscle on each side of any individual animal was not significantly different, the result given in Table 1 is the average value for each animal and represents the mean of 12 measurements—6 on each psoas muscle.

In the D- rabbits the rate of calcium uptake by the SR

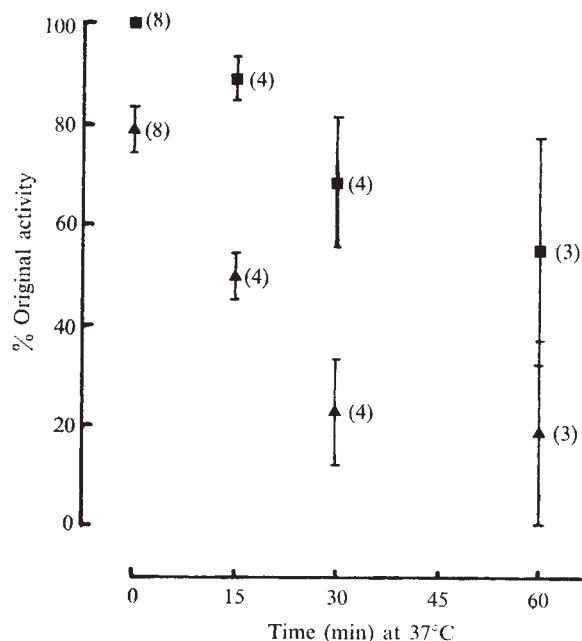


Fig. 1. Percentage of initial rate of calcium uptake by SR after incubation at 37°C. The results are expressed as a mean $\pm$ s.e.m. of the rate of calcium uptake of the paired control when first measured. Numbers of animals are given in brackets. ■, D+; ▲, D-.

was lower in all experiments than in the D+ controls. The mean difference between the pairs of D- and D+ rabbits is significantly different from zero ($t = 7.27$; $P < 0.001$), and the difference between the mean rates of calcium uptake for the two groups is also significant ($t = 2.25$; $0.05 > P > 0.02$). In some experiments the SR samples were incubated for varying times at 37° C and the rates of calcium uptake subsequently measured (Fig. 1). The SR from the D-

animals lost its ability to concentrate calcium more rapidly than that from the D+ controls.

No consistent differences were observed by the linked spectrophotometric assay of Horgan, Tume and Newbold¹⁰ in the rates of either the Mg^{2+} or the Mg^{2+} - Ca^{2+} stimulated ATPase between the SR isolated from the two groups of animals. In an initial repletion experiment three oral daily doses of 100 IU of Vitamin D₃ were given to one of a pair of D- rabbits. The rate of calcium uptake by the SR from the repleted rabbit was higher than that of the D- rabbit (1.39 as against 1.09 μ mol Ca per min per mg SR protein). The activities of the Mg^{2+} and Mg^{2+} - Ca^{2+} stimulated ATPase were again similar.

In rabbits up to the age of 8 d it has been shown that both muscular activity and age affect the rate of calcium uptake by SR¹¹. The subsequent changes until maturity are not known. In our rabbits the animals in each pair were exactly the same age and this showed no apparent relation to the rate of calcium uptake by the SR; there was also no clear difference in their spontaneous muscle activity although the D- animals were weak and hypotonic. We consider that the difference in calcium uptake between the D- and the D+ animals is due to an effect of vitamin D deficiency on the muscle itself rather than on its nerve supply. In rats denervation may increase (rather than decrease) the rate of active calcium transport by the SR of slow muscle, but not of fast, to which group the psoas belongs¹².

How vitamin D deficiency affects SR is not known. As no differences were seen in the Mg^{2+} and Mg^{2+} - Ca^{2+} stimulated ATPase activities of the SR from the D- and D+ rabbits, the observed alteration in the rate of calcium uptake may be a result of changes in the calcium transport coupling system¹¹⁻¹⁴, and similar 'inefficient' SR has been isolated from dystrophic muscle¹⁴.

There are clearly many possible calcium dependent mechanisms in muscle upon which vitamin D might have an effect. Our results provide support for the inclusion of muscle as another target organ for vitamin D.

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Site and mechanism of water vapour uptake from the atmosphere in ixodid ticks

UPTAKE of water vapour from subsaturated atmospheres has been demonstrated in a variety of terrestrial insects and mites but no previous effort to identify the site and mechanism has been completely conclusive. The tracheal system, the rectum and the general body surface have variously been proposed in specific studies.

Noble-Nesbitt¹ found that occlusion of the anus of the insect *Thermobia domestica* (Packard) prevented recovery of an experimentally imposed body water deficit and he suggested that the site of vapour sorption is in the rectum. The tick *Dermacentor variabilis* (Say), however, was not impaired in this ability by blocking the anus^{2,3}.

The present investigation deals with the site of water vapour uptake in the ixodid tick *Amblyomma variegatum* (Fabricius) and considers a possible uptake mechanism. Unengorged adults, both males and females, were used between 8 and 20 weeks after ecdysis. During this period there were no waste products; weight and water content (60% of total weight on the average) showed no significant changes at constant conditions of 20° C and 93% relative humidity (r.h.) This humidity was well above the critical equilibrium humidity (found to be between 80% and 85% r.h.) of the adults. At 20° C, ticks could be handled without using carbon dioxide anaesthesia.

Ticks exposed to 33% r.h. and 20° C for 4 weeks lost 17%-19% of their initial weight or 28%-31% of their initial water mass. Subsequently, in groups of 10 ticks each, the following parts of the body were covered with paraffin wax (melting point 42° C-44° C): the anus, the complete dorsal surface and the mouthparts. A control group of 20 ticks remained without paraffin treatment. During the ensuing exposure to air of 93% r.h. and 20° C the body water mass increased at $3.0 \pm 0.7\%$ d⁻¹ in the control ticks, $3.4 \pm 0.6\%$ d⁻¹ in those with the anus covered, $3.1 \pm 0.8\%$ d⁻¹ in those with the dorsal surface covered; those ticks with the mouthparts covered lost $0.3 \pm 0.1\%$ d⁻¹ of their body water for a period of 4 weeks.

The effect of covering the mouthparts is also evident in hydrated ticks in equilibrium with 93% r.h. Of a group of 20 ticks, those untreated were able to maintain equilibrium weight in air of 93% r.h. and 20° C; but those with the mouthparts covered lost $0.2 \pm 0.1\%$ of their body water each day during an observation period of 7 weeks.

To show that the application of paraffin wax and the contact between the mouthparts and the applied wax had no adverse effects on water uptake, the following experiments were performed. In one group of ticks, dehydrated as above, only the distal half of the mouthparts was painted with paraffin wax such that both the dorsal space between the chelicerae and the lateral spaces between the hypostome and the chelicerae provided paths between the surrounding air and the buccal cavity. These ticks were able to increase their body water, comparable with that in the control group, at a rate of $2.9 \pm 0.5\%$ d⁻¹ in air of 93% r.h. and 20° C. When the wax was removed manually from 10 ticks that had suffered loss of weight with their mouthparts sealed

with wax, vapour sorption commenced immediately in each. Moreover, in most ticks with sealed mouthparts, beginning after approximately 4 weeks the wax cover progressively softened from the inner surface (perhaps because of ejected saliva), gradually detached itself from the mouthparts and after about 5 weeks could be removed in one piece. Accompanying this process, the rate of gain of body water mass increased gradually from $\sim 0.5\%$ d^{-1} in the early stages of loosening to $\sim 3.0\%$ d^{-1} after the removal of the wax cover. From these experiments we can conclude that blockage of the mouth prevents net water gain from the atmosphere in *A. variegatum*.

That water vapour actually passes through the mouthparts was shown in an experiment which did not require blockage of the mouthparts with paraffin wax. For this purpose the mouthparts of a dehydrated tick were inserted through a wax-coated parafilm membrane up to the base of the palps. This membrane was clamped between the open ends of two glass tubes (inner diameter 5 mm, length 20 mm) such that the mouthparts projected into one tube and the rest of the body into the other. The contact line between the two tubes and the penetration point of the mouthparts through the membrane was carefully sealed with melted paraffin wax. The opposite ends of the glass tubes were closed by parafilm membranes coated with paraffin wax. By injecting the needle of a microlitre syringe through these parafilm membranes, 1 μ l of saturated KNO_3 solution (which provided a humidity of 93% at 20° C) was placed on the wall of each glass tube, adjacent to but not touching the tick. The membranes were subsequently repaired. After 28 h the drop of KNO_3 solution adjacent to the mouthparts had changed to a moist crystal of KNO_3 , whereas that in the tube containing the rest of the body seemed to be unchanged. The possibility that vapour inadvertently escaped to the surrounding air was completely precluded by holding the assembly in a container over KNO_3 solution. This experiment brought the same result when repeated with five different ticks. The rate of decrease in the the volume of the solution adjacent to the mouthparts was estimated to equal in magnitude the rate of net water gain from air of 93% r.h. and 20° C that had been measured in similarly dehydrated ticks.

Active uptake of water vapour, either to acquire or to maintain equilibrium water mass, takes place through the mouthparts in the ixodid tick *A. variegatum*. Since similar results have been obtained when these experiments were performed on adults of *Rhipicephalus bursa* (Can. et Fanz.), *Hyalomma anatolicum excavatum* (Koch) and *Hyalomma schulzei* (Ol.), it may be suggested that active uptake of water vapour proceeds through the mouthparts in all ixodid ticks.

There is some evidence for a significant role of the saliva in the mechanism of vapour uptake as shown by the following observations. Severely dehydrated adults of *A. variegatum* (desiccated to $50.6 \pm 11.3\%$ of their initial water mass) have been observed to secrete through the mouthparts a clear fluid which accumulated between the hypostome and the palps and quickly dried to a white crystalline solid in the desiccation chamber, but when these ticks were transferred to humidities greater than the critical equilibrium humidity the crystalline solid dissolved in absorbed water and the liquid was subsequently imbibed. These crystals occurred only between the hypostome and the palps and so the passage to the mouth remained open. Moreover, the clear fluid could be observed in the buccal cavity and the salivary ducts of all ticks (regardless of water status) after removal of the chelicerae and the dorsal part of the capitulum.

The crystalline solids, described above, were collected from the mouthparts of dehydrated ticks and exposed to a series of different humidities. The substance remained unchanged in humidities less than 75%; at 75% r.h. it became moist

without dissolving, above 80% r.h., however, it took up substantial amounts of water within a few seconds and finally dissolved. Recrystallisation occurred when the dissolved substance was transferred to relative humidities less than 75%. It should be noted that the lower limit of water uptake by the crystalline solids corresponds with the critical equilibrium humidity of the species.

Preliminary chemical analysis of the crystalline substance has identified high concentrations of sodium and potassium; anions have not yet been identified. To obtain greater amounts of secretion from the mouthparts 0.5 μ l of a 3% isotonic solution of pilocarpin was injected into unengorged adults which were in equilibrium with 93% r.h. at 20° C. This immediately led to copious salivation. The secretion obtained was, however, different from that described above. It lost water only very slowly at low humidities and did not exhibit the dramatic water uptake at high humidities. Furthermore, it contained an immiscible viscous, milky cement substance which has been described for ticks in connection with attachment to a host.

This study shows that active uptake of atmospheric water vapour in ixodid ticks proceeds through the mouth. It may well be related to hygroscopic properties of salivary secretion. Resorption of the absorbed water could possibly take place in the gut, although the remarkable abilities of the salivary glands in handling water⁴⁻⁸ should be kept in mind. Further experiments on the uptake of water vapour in ticks are in progress.

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Is texture-density contrast an inhibition or an adaptation?

MACKAY's recent experiments on texture-density contrast¹ are an interesting contribution to the current, rapidly-growing literature on 'feature-specific inhibition' in the visual system. There have been suggestions, based mainly on psychophysical evidence, that there is mutual inhibition between 'channels' selective for binocular disparity², orientation^{3,4} and spatial frequency^{5,6}. It is known that many neurones in the visual cortex respond selectively to particular values on these three dimensions⁷⁻⁹ (as well as others, such as direction and velocity). Our knowledge of lateral inhibition at earlier levels in the visual pathway indicates that mutual inhibition between

such multi-channel neurons would account for a wide range of visual effects.

Following a previous suggestion¹⁰, MacKay has proposed a new type of neurone, responding as a monotonic function of texture density, with inhibition between such units playing an important perceptual role in enhancing local density gradients (such as the edge between two surfaces of different material, or at different distances). Before accepting the existence of 'texture-density contrast' as an important phenomenon, however, we must be sure that MacKay's observations cannot be explained in terms of known effects.

Successive contrast between gratings or textures of different densities (the 'spatial frequency shift') is well documented¹¹⁻¹³. After inspection of one pattern, a subsequently viewed finer pattern looks even finer, a coarser one even coarser. It is particularly important when doing experiments aimed at showing simultaneous contrast to show that the effects obtained are not simply the after effects of adaptation^{14,15}. For example, when viewing MacKay's demonstrations¹ (Fig. 5 of ref. 1), if fixation drifted onto the background, even for a second or two, an after effect would be expected when the test pattern was refixated.

MacKay himself reported that the contrast effect was enhanced by moving the figure up and down while the eye was held stationary. This of course suggests an explanation in terms of adaptation, as fixation was alternating between the background and test patterns. Because MacKay presented no formal data, and little information on viewing conditions, it is impossible to assess the likely extent of adaptation in his experiments.

Turning to his demonstrations, the reader is faced with some difficulties in judging the phenomenon. Figure 5a cannot properly be assessed, as it contains a serious artefact. The two outermost lines in the left half of the test grating are in contact with lines in the background grating, and so actually are wider! Another error occurs in Fig. 2a, which contains three regions of supposedly equal frequency. On taking a ruler to it, one finds 8 cycles cm^{-1} in the central and right-hand regions, but about 8.5 cycles cm^{-1} in the left-hand region. This difference of 6% is in the same range (5-10%) as the effect claimed. The reader cannot tell whether it is an error in photographic reproduction, or was present in the original display.

I have obtained reports from several observers, who agreed that the dot pattern of Fig. 5b gave a noticeable contrast effect on immediate viewing, but most were unable to make a judgment on the random texture of Fig. 5c.

About a year ago, I did some experiments also aimed at showing spatial-frequency contrast, with stimuli very similar to MacKay's Fig. 5a. The background patterns were vertical, high-contrast sinusoidal grating photographs of 3.5 and 7 cycles per degree. The two test patterns were of much lower contrast, at 5 cycles per degree, each subtending $1.5^\circ \times 1.5^\circ$, one vertically above the other, separated by a grey strip 0.25° wide. They were presented in a tachistoscope, with the test patterns either both vertical or both horizontal, for 0.5, 1, or 2 s with instructions to fixate a mark centred between the test gratings, and to report whether the upper or lower test grating appeared to have the broader bars and/or wider spacing.

In the first experiment, the background pattern was left unchanged over a sequence of 120 trials for each of four subjects. The result for vertical test gratings was an average of 78% reports in favour of contrast, while for the horizontal test, only 57% (where 50% represented chance).

Although the trials were brief, specifically to avoid adaptation, I then realised that adaptation to the background could have been building up over trials. Five more subjects were run, with 0.5 or 1 s presentations and the background patterns were interchanged after each trial to balance out any spatial adaptation. The results in this series were much more

variable, and did not deviate significantly from chance (48% with test vertical, 37% with test horizontal). I concluded that a spatial-frequency, or texture contrast effect did not exist, when adaptation was properly controlled for.

In view of MacKay's demonstration with dot textures, it now seems possible that a texture-contrast effect may exist in some circumstances. Much more controlled experimentation is required, however, to demonstrate that it has any generality, and that it is not due to the after-effects of adaptation to the background pattern. Of course, the spatial-frequency shift (after effect) may itself play a role in enhancing a texture discontinuity, but it does not need a new type of neurone or process to account for it.

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PROFESSOR MACKEY REPLIES: Dr Georgeson¹ could easily have disposed of most of his doubts.

The test strips in my demonstrations of simultaneous space-frequency contrast² had a subtense of between 1° and 2° . There is no difficulty in keeping one's fixation on the horizontal midline of such a broad strip, yet an effect of about 10% is then measurable even with Fig. 1, which has no background lines at the ends of the strip. Slight vertical oscilla-

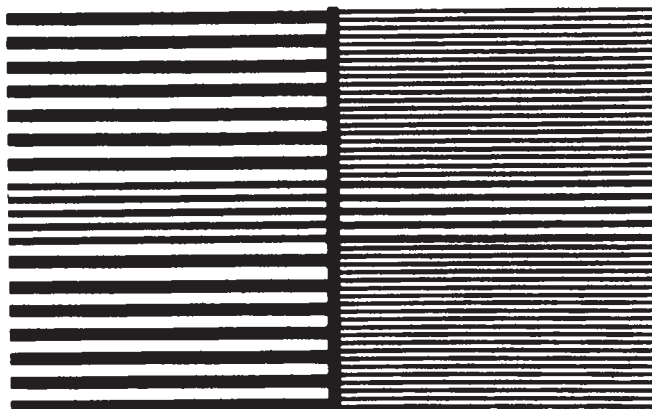


Fig. 1 Modified form of Fig. 5a (ref. 2).

tion of the figure enhances but is not at all essential to the effect.

The effects for some O's are stronger immediately on viewing than after a minute or so of inspection. This adaptation

is opposite to that which might be expected on Georgeson's hypothesis.

What Georgeson describes as a 'serious artefact' is easily evaluated. With movable test strips, the contrast effect is seen regardless of their location relative to the background stripes, and Fig. 1, free of the allegedly 'serious' artefact, is just as effective as my original figure (Fig. 5a ref. 2).

The distortion of the line-spacing on the left of Fig. 2a of ref. 2 is regrettable; but it need not prevent the reader from checking the points made, which depend on (i) comparing the perceived spatial frequency of the central panel with that of either side panel, (ii) observing the effects of masking the transition regions, and (iii) comparing Fig. 2a with Fig. 2b. (i) Only the left-hand panel was distorted; yet covering over this panel as far as the transition region (or indeed the whole panel) does not abolish the illusory frequency contrast between the other two panels.

(ii) Masking the transition regions, on the other hand, does abolish the illusion.

(iii) In Fig. 2b, the centre frequency of the middle panel as printed (8.25 cycles cm^{-1}) is still higher than the average for the other two panels (7.75 cycles cm^{-1}); yet it is perceived as matching them when the transition regions are visible, and not when they are masked.

In view of all this, Dr Georgeson's own negative findings with different stimuli under different conditions scarcely justify his conclusion.

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Mid-Precambrian prokaryotes(?) from the Belcher Islands, Canada

SPECULATION about the Precambrian biosphere and about evolutionary history is slowly being substantiated and modified by new palaeontological evidence from many regions. I present here more such evidence—a preliminary report of the discovery of a well preserved stromatolitic microflora from cherty dolostones of the Belcher Supergroup in Hudson Bay. This microflora may provide important information about the stage of algal evolution about 2,000 Myr ago, and about stromatolite genesis. Furthermore, the well preserved cellular biofabric facilitates description of the development of primary stromatolitic microstructure permineralised *in situ* by silica, and its subsequent modification (Fig. 1a) by carbonate diagenesis into a secondary microfabric, which is important in stromatolite taxonomy.

This fossil microflora contains the oldest known, well preserved palmelloid algal colonies, and their mode of growth and occurrence in stratiform and domal stromatolites confirm that non-filamentous algae are important in the formation of Aphebian stratiform stromatolites. Cells have well differentiated morphological details, such as protoplast, cell wall, and a laminated envelope (Fig. 1). Moreover, two of the taxa in these colonies contain dark inclusions in the protoplast. In the Bitter Springs microflora¹ and elsewhere similar structures have been interpreted as nuclei, and the fossils as eukaryotic, and the same evidence could be used for a similar interpretation of the Belcher material. On the other hand, the dark

bodies may be granular inclusions of prokaryotes, or plasmolytic artefacts² of prokaryotes or eukaryotes, or possibly diagenetic relics.

Microfossils and possible microfossils are already known from the Belcher Islands^{3,4}. Those reported here occur in black chert layers and pockets in laminated, stromatolitic carbonates of the Kasegalik and McLeary Formations in the lower part of the Belcher Supergroup^{5,6}. These rocks form part of an Aphebian miogeoclinal sequence of sedimentary and volcanic rocks belonging to the Belcher Fold Belt, part of the larger Circum-Ungava geosyncline, the principal phase of deformation of which embraces the period of the terminal Aphebian Hudsonian orogeny. The latest available isotopic age determinations⁷, based on the Rb-Sr whole-rock isochron method ($\lambda^{87}\text{Rb} = 1.39 \times 10^{-11} \text{ y}^{-1}$), indicate a minimum of 1800 Myr for the sequence yielding the microfossils. Basement is not exposed in the Belcher Islands, but correlative units along the Manitounuk-Nastapoka arc to the east lie on Archaean basement which is 2,500 Myr old⁸. The general geology and geological history of the Belchers has been described by Jackson^{4,6}. A more detailed study of the stratigraphy, sedimentology and palaeontology of the Belcher rocks is in progress (R. T. Bell and H. J. H., in preparation); this should lead to a better understanding of the Aphebian record there.

The new Belcher microfossils were obtained from the upper parts of the Kasegalik Formation on an unnamed island at the northern end of Churchill Sound (56°29.5'N

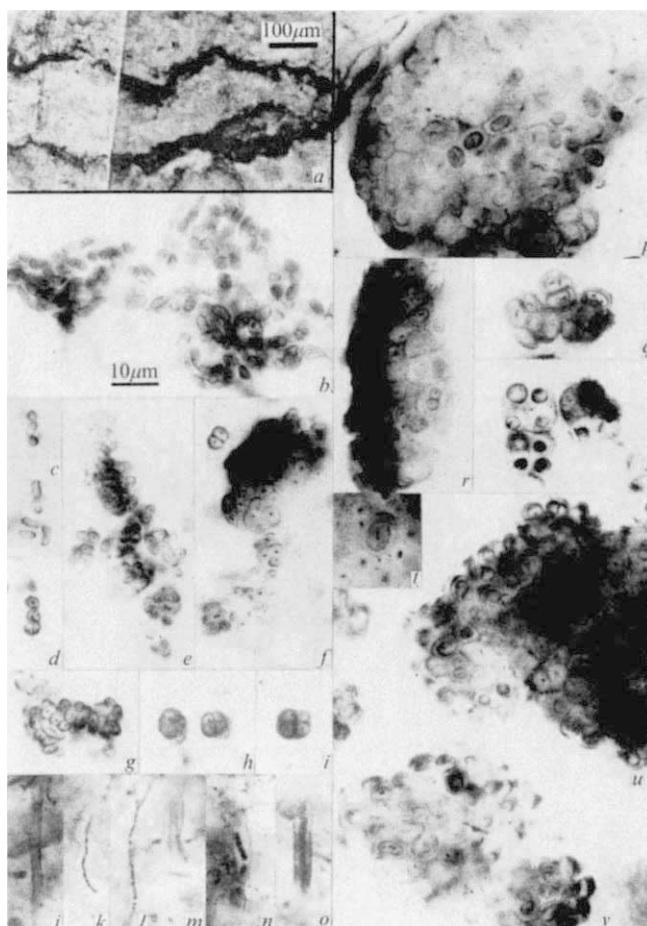


Fig. 1 Optical photomicrographs of stromatolitic lamellae and microfossils in thin sections from the Kasegalik Formation of the Belcher Islands. Compare the effects of carbonate diagenesis on dark, organic lamellae in the left third of (a) with the unaffected parts in silica matrix to the right. Magnifications for (b)–(v) are identical, and are ten times that for (a).

79°30.2'W), and from several levels within the McLeary Formation on Flaherty Island, along the south shore of Eskimo Harbour. Both formations show evidence of peritidal deposition. The geological setting of the stratiform and domal algal mats of the two formations, yielding the palmeloid colonies illustrated in Fig. 1, is probably a large sebkha/algal flat/shallow basin complex, as attested by abundant desiccation features, laminated structure and the presence of halite and sulphate casts⁹.

In mode of preservation, level of organisation and palaeo-environmental setting, the new Belcher assemblage compares with other Precambrian assemblages such as those in the Gunflint or Bitter Springs cherts, but it is distinguished from them by its relatively small diversity of species. This probably reflects its mature biocoenotic character of uniform, environmentally restricted algal mats, rather than a lack of diversity of organisms existing about 2,000 Myr ago.

The half-dozen new microfossils and the stromatolites will be described formally elsewhere after further investigations. For this report the microfossils are assigned to the following categories. (1) Loose aggregates of oblong bacterioid cells without inclusions and without envelopes, 1.5–2 μm wide and 3–4 μm long (Fig. 1b and c). (2) Colonial unicells in loose clumps; composed of spheroidal cells averaging 2 μm in diameter, with single dark inclusion and without envelope (Fig. 1d–i); some cells show evidence of division. (3) Compact, coherent colonial clusters and crinkled, pustulose stromatolitic layers composed of ellipsoidal to ovoidal cells with well differentiated protoplast, 3–5 μm in diameter, dark inclusions 0.5 μm across, cell wall, and a thick sheath which may be laminated around some cells and with an external form which is polyhedral because of close packing. (4) *Eomycetopsis*-like tubes 3–4 μm across, possibly representing empty sheaths of filamentous algae (Fig. 1j). (5) *Gunflintia*-like trichomes less than 1 μm across and up to 40 μm or more long, made up of a chain of dark spheroidal bodies (Fig. 1k and l). (6) Filaments composed of a chain of dark bodies 1 μm across, surrounded by a sheath 3–4 μm in diameter (Fig. 1m–o). This type may represent more nearly complete individuals composed of forms (5) and (6).

The biological affinities of these fossils are not known, although an algal origin for the colonies seems the most plausible. Nor is it established whether the different unicellular types are really different organisms or whether two or more represent different ontogenetic or degradation stages of the same species.

Of special interest are the types with dark bodies (Fig. 1d–i; p–v), with well defined protoplasts. Similar inclusions, though in larger microfossils, from the Late Precambrian have commonly been interpreted as nuclei¹. Without these bodies, the size range and morphology would invite comparison with such modern blue-green algae as *Chroococcus*, *Microcystis*, *Entophysalis* and *Gloeocapsa*. Recent experimental evidence indicates that such dark internal bodies may be entirely plasmolytic artefacts². However, this does not necessarily mean that all microfossils with internal dark spots so far described should be reassigned to the prokaryotes: it provides an alternative explanation. Other possibilities are that the structures are remains of metabolically-functional organelles, vacuoles, intracellular symbionts¹⁰, or diagenetic artefacts. Present evidence, therefore, is inconclusive as to whether the structures are eukaryotic or prokaryotes preserved at different stages of plasmolysis. Evidence against the eukaryotic nature of the microfossils is the small size of the cells, and the lack of other unquestionable eukaryotes from rocks older than 1,000–1,200 Myr, although microfossils with attributes suggestive of eukaryotic organisation have been reported from the Aphebian^{4,11}. Nevertheless, it is possible that the evolution of larger, chlorophytic algae with normal eukaryotic organisation began with the development of nuclear bodies and

organelles in primitive, cyanophyte-sized, ancestors at least 1,800–2,000 Myr ago; the Belcher microfossils would represent such remains.

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Erratum

Unfortunately during preparation for the printer the axes of Fig. 2 were wrongly labelled in the letter "Ion fluxes in disk membranes of retinal rod outer segments" by W. T. Mason, R. S. Fager, and E. W. Abrahamson (*Nature*, **247**, 562; 1974). The corrected figure is now shown.

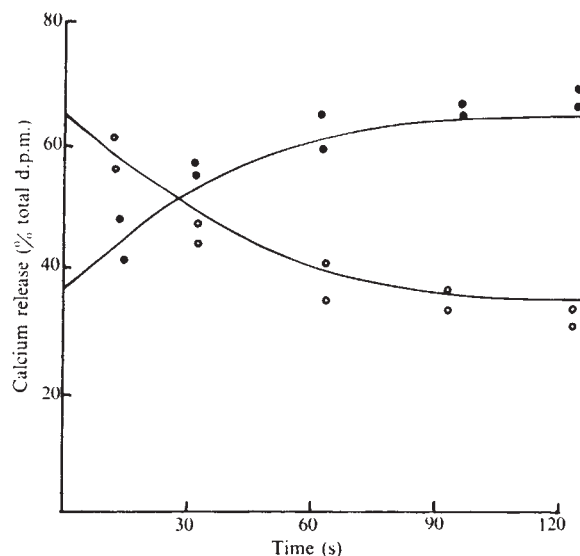


Fig. 2 Light-induced calcium release by sonicated bovine photoreceptor membranes over a time course of 120 s bleaching. Filled circles indicate percentage of Ca^{2+} in the extradisk space and open circles the percentage of Ca^{2+} in the intradisk space.

book reviews

Solid state

Theoretical Solid State Physics. By W. Jones and N. H. March. Vol. 1, *Perfect Lattices in Equilibrium*: pp. xvi+680; vol. 2, *Non-Equilibrium and Disorder*: pp. xv+681-1301. (Interscience Monographs and Texts in Physics and Astronomy.) (Wiley: London and New York, September 1973.) Each volume £14.75.

An introduction to Solid State Physics and its Applications. By R. J. Elliott and A. F. Gibson. Pp. xx+490 (Nature-Macmillan Physics Series) (Macmillan: London and Basingstoke, February 1974.) £5.95.

TEXTS on solid state physics are numerous. It is therefore pleasing to note the appearance of two new works for which substitutes are not already available. The two volumes by Jones and March give a comprehensive coverage of a large area of theoretical solid state physics and are directed toward professional theoreticians and postgraduate students. In contrast, the book by Elliott and Gibson is aimed at the undergraduate in the general area of material science and brings together the theory of solids and device applications.

The first volume of Jones and March includes band theory and crystal symmetry, collective effects in solids, lattice waves, spin and orbital magnetism, interactions of particles with a crystal. The second volume covers transport, optical properties, superconductivity, polarons and excitons, defects and disordered systems. Detail is developed in numerous appendices, and a set of problems and a basic bibliography are given.

In general the material is thoroughly covered and developed as far as more important current research papers. Techniques of many body theory are introduced in the text and developed in appendices, the fullest discussion being centred round the Coulomb interaction, though interactions involving phonons are also treated. The consequences of symmetry are also extensively discussed and an 83-page appendix on group theory is provided.

The chapter on band theory, typically, bridges the gap nicely between the limited treatment of a single volume treatise and the specialised monograph. Inevitably, for some sections, readers will prefer other texts. For an introduction to plasma effects and the electron-phonon interaction, I find Pines's *Elementary Excitations in Solids* clearer,

although the present work does, with advantage, include detailed many body theory. Regarding the electron-phonon interaction, Pines's initial general survey establishes greater unity than the present separate discussions under phonons, transport and superconductivity.

A nice perspective is developed in the chapter on magnetism. The starting point is the general interacting electron Hamiltonian which leads to a study of the Heisenberg and Hubbard models and a clear appreciation of the approximations involved. The discussion of spin waves and magnetic phase transitions relates to neutron scattering theory which appears in the useful chapter on particles as probes for the study of solids.

Some topics have of necessity been omitted (polariton theory is one), and others chosen for only brief coverage: for example, infrared absorption under optical properties and the Kondo effect in the chapter on disorder. Otherwise this chapter is a full survey of a wide field of much recent activity.

This work, an extensive account of present understanding of solid state theory, is a valuable addition to the literature. The authors are to be commended for undertaking this mammoth task.

The opening two chapters of Elliott and Gibson discuss basic ideas of crystal structure and excitations. The reciprocal lattice is dealt with only briefly and the beginning student may need to refer to a more discursive text. The next two chapters cover lattice vibrations and electrons in bands. The basic theory of lattice vibrations is introduced, followed by neutron scattering results for various crystals, starting with the 'simple' rare-gas crystal and developing through more complicated types. The classification and comparison over a short space is particularly effective. Being of major importance in applications, the fifth chapter treats imperfections in some detail.

A survey of photon absorption over the range 10^2 – 10^{-3} eV in the section on optical properties puts the various absorption processes into perspective. The chapter finishes with optical emission and non-linear optics. The following two chapters deal with optical and microwave properties of free carriers, and transport which includes superconductivity and its applications. The ninth chapter is entirely device oriented (semiconductor junction devices) and the

book concludes with theory and applications of magnetic materials. Problems and answers are provided.

Many students will find this book too demanding because of its conciseness in presentation of some basic theory. Hence it is unlikely to replace books such as those of Kittel or Dekker as a basic teaching source, although I expect it to be used widely in conjunction with these. On the other hand, the terseness in style does give the reader an excellent connected impression of the subject. The wood stands out clearly but the student may have to refer elsewhere for a delineation of some of the trees.

J. A. BLACKMAN

Unicellular eukaryotes

Protozoology. By Karl G. Grell. Pp. viii+554. (Springer-Verlag: Berlin and New York, 1973.) 107 DM; \$43.90.

IN former times protozoology was considered to be a suitable subject by which students could be introduced to biology, presumably because, being single-celled animals, protozoa were thought to be simpler than so-called higher organisms, and from an evolutionary point of view they seemed primitive. Extensive courses describing many types of protozoa were commonly given and a number of solid textbooks were available, mainly in German, and largely, of course, of a taxonomic nature. Now all that is changed and few university courses treat protozoology in depth.

Protozoa are, however, important objects of study from a number of points of view. They provide admirable experimental material for many types of work, in genetics, nutrition, sexuality, behaviour, and so on. They combine the advantages of being single cells and also of being undoubted eukaryotes, that is, basically the same as cells of higher organisms. They can be used as models for the formulation and experimental testing of theoretical systems of cell organisation involving nuclei, various cytoplasmic components and the external surface. Finally, some protozoa cause exceedingly unpleasant diseases, some still very common and almost uncontrolled in large regions of the world.

The book by Karl Grell is, therefore, very welcome as it is the only one which attempts to discuss the subject in a modern context. Only about a quarter is given to taxonomy, the remainder

being devoted to a general treatment of morphology, cytology, genetics, nutrition, behaviour and so on. Sometimes indeed it seems that protozoology is really a non-subject, a miscellaneous assortment of different types of study on an extraordinarily diverse collection of organisms.

The excellent section on genetics, for example, is based mainly on studies with *Chlamydomonas* and *Paramecium* and includes a great variety of topics. The only feature which stands out is the relatively large amount of attention given to non-Mendelian heredity, involving chloroplasts, mitochondria, kappa particles and other odds and ends. A particularly detailed account of work on Foraminifera is given, reflecting the special interests of the author. On the other hand, important parasitic protozoa, such as the malaria parasites, trypanosomes, *Leishmania* and so on, seem to get rather less attention than they deserve.

The book is extremely well printed and beautifully illustrated, and can be thoroughly recommended to those who can afford it. English-speaking readers must be grateful to the author for writing the book in their language, (earlier versions were in German), though some, may find themselves, as I was, at times irritated and even baffled by some linguistic idiosyncrasies. On such occasions a little knowledge of German still helps.

G. H. BEALE

Atmospheres of planets

Physics of Planetary Ionospheres. By Siegfried J. Bauer: Pp. viii+230. (Physics and Chemistry in Space: vol. 6.) (Springer-Verlag: Berlin and New York, 1973.) 78 DM; \$35.10.

Dr Bauer has worked in the field of planetary ionospheres for more than fifteen years. Working at the NASA Goddard Space Flight Center he has been closely associated with many of the recent developments in the subject.

His book, intended as an introduction, develops logically from a discussion of neutral atmospheres, ionising radiations, chemical processes and plasma transport. In this respect it follows the pattern of many recent books on ionospheric physics. It differs from these in that at each stage it extends the discussion to the planets, particularly Mars, Jupiter and Venus. The space devoted to the other planets seems to be related (quite reasonably) to the thickness of their atmospheres.

In later chapters Dr Bauer discusses models of planetary ionospheres, wave propagation and experimental techniques. The treatment of the last two subjects is rather synoptic and the student may find it necessary to consult

the many references for enlightenment. The last chapter gives details of the observations of planetary ionospheres and includes electron density profiles for Mars and Venus measured by the Mariner spacecraft. It is perhaps surprising that only nine pages are devoted to the discussion of these interesting results.

This book has many excellent qualities: the diagrams, tables and general layout are attractive and there is a comprehensive list of up to date references. The author is familiar with the Russian literature and his explanation of some of the Russian terminology will be useful to many. It has become standard practice in the United Kingdom to use SI units in scientific texts. Dr Bauer uses a mixture of c.g.s. and practical units but I found this no great disadvantage.

The author intended his book "to be an introduction to the subject for a new generation of space scientists and a compendium for the old". In my view he has been largely successful. But even at today's prices fifteen pounds is inordinately expensive for a book of 230 pages.

A. R. W. HUGHES

Atomic time

The Measurement of Frequency and Time Interval. By L. Essen. Pp. v+55. (National Physical Laboratory: Teddington, December, 1973.) £1.

THE rapid development of increasingly precise methods for the generation of specific (atomic) frequencies and their measurement has had a resounding impact on a vast range of theoretical disciplines and practical applications. Accurate control of frequency is a fundamental requirement of radio and it makes possible, for example, the Doppler measurement of rate of change of distance required in navigation and space research. Since 1967 the SI unit of time has been defined in terms of the frequency of a particular transition of the caesium atom, thus giving rise to the integrated reference time scale of International Atomic Time (TAI), obtained by the continuous summation of time intervals deduced from measures of frequency. Coordinated Universal Time (UTC), an approximation to UT1 (or GMT) and which differs from TAI by an integral number of seconds, is now the basis for civil time in most of the world; it provides direct synchronisation to about a millisecond, but individual atomic clocks may be compared with much higher precisions (tens of nanoseconds) making possible, for example, the almost direct verification of the predicted relativistic dependence of clock rate on speed and gravitational potential. A rather more practical appli-

cation is Loran C for navigation.

This monograph, written by the acknowledged pioneer in the field, describes the basic principles and practical applications of the use of atomic transitions (or spectral lines) as standards of frequency. The treatment is simple and straightforward; the descriptions of the methods used at the National Physical Laboratory, given with explanatory diagrams and photographs, are exceptionally clear even to a layman in the field. Determinations of precision and stability are fully covered. Understandably the main emphasis is on the caesium definitive standard, but Dr Essen also covers working standards (commercial standards, which are portable, are now available with precisions of the order of 1 part in 10^{11}), standard frequency radio transmissions, and the techniques of the comparison of frequencies. His final chapter is devoted to the design and performance of resonance (particularly cavity resonator) frequency meters. It thus provides invaluable background information for the many concerned with the applications of these fascinating developments. But it seems that much of the text was written in 1972 so that those more directly interested in the latest work, especially at laboratories other than NPL, should refer to the technical journals.

Dr Essen devotes one short chapter to atomic and astronomical time, and refers briefly to the modified form of UTC introduced as from January 1, 1972. Although he refrains from saying so, it must now be generally agreed that the adoption in 1956 of the ephemeris second as the SI unit of time was a grave mistake: the astronomers were over-optimistic as regards the practical and theoretical difficulties of its determination, whereas the physicists were not agreed as to which of several possible atomic transitions should be used as the standard. Apart from confusion and change, the second of Ephemeris Time (introduced purely as a theoretical astronomical time scale) is not an approximation to the current value of the second of UT (which varies with the speed of rotation of the Earth), so that the preservation of its value in the 1967 unit results in an unnecessarily large rate of departure of IAT from UT. This has perhaps led Dr Essen to imply that the introduction of leap-seconds into UTC on the preferred dates of 30 June and 31 December would enable the modified time signals to be maintained within ± 0.7 s of UT1—so drastically contradicted on January 1, 1973.

In spite of this misunderstanding (due mainly to the shortcomings of CCIR Report 517) Dr Essen has a right to be proud of his contributions and of his readable and informative monograph.

D. H. SADLER

Attacking viruses

Selective Inhibitors of Viral Functions. Edited by William A. Carter. Pp. 376. (CRC: Cleveland, Ohio, 1973.) \$39.95.

It is the opinion of many animal virologists that, with one or two isolated exceptions, the successful treatment of virus diseases with specific anti-viral drugs is a subject which falls into the pipe-dream category. Optimists can be found, however, and the editor of this multi-author work clearly defines himself as one in his introductory remarks. He has assembled here a collection of reviews concerned with both laboratory and clinical aspects of specific anti-viral agents together with accounts of host reactions to viral infections and of immunisation against viral infections.

The natural recovery process from acute viral infections is dealt with comprehensively and concisely by W. E. Stewart. He clearly points out that it is still difficult to assess the relative importance of circulating antibody, cell-mediated immunity, interferon, and non-specific defence mechanisms. In the chapter on immunisation against viral infection, L. W. Marshall briefly discusses theoretical aspects and describes the viral vaccines and passive immunisation procedures currently used in human medicine.

A speculative article "Active Sites of the Animal Viruses: Potential Sites of Specific Chemotherapeutic Attack" by W. M. Mitchell follows. I feel that this author's enthusiasm has rather carried him away from reality, and that non-specialist readers might imagine, for example, that the synthesis of biologically active human interferon by solid-phase methods was just around the corner. This impression, is, however, corrected by a chapter on interferon structure by R. Weil and F. Dorner. Other chapters on the effects of interferon, the molecular requirements for interferon induction, interferon induction by polynucleotides and non-nucleotide interferon inducers are contributed by E. De Clerq and W. E. Stewart, M. D. Johnston and D. C. Burke, A. K. Field and E. De Clerq respectively. It is apparent that one of the most important aims of interferon research—its purification to homogeneity—is proving an intractable problem.

The remainder of the book is concerned largely with synthetic and semi-synthetic anti-viral compounds and antibiotics. Amantadine hydrochloride and related compounds, arabinosyl nucleosides and nucleotides, and halogenated pyrimidines are discussed, largely in a clinical context, by C. E. Hoffmann, L. T. Chien, F. M. Schabel and C. A. Alford, and J. Sugar and H. E. Kaufman, and comprehensive accounts of the anti-viral properties of thiosemicarbazones, guanidine

dine and 2-(α hydroxybenzyl) benzimidazole, rifamycin SV derivatives, and streptovaricins are given by W. Levinson, L. A. Caligiuri and I. Tamm, B. Moss, and D. M. Byrd and W. Carter. The book concludes with a short article by P. M. Pitha on the possibility of inhibiting virus growth by the use of viral genome analogues.

The quality of the contributions in this volume is generally high, although one or two early chapters show evidence of sloppy proof reading and reference checking. The book can be recommended as an up to date account of its subject, the comprehensive bibliographies which conclude most chapters being particularly useful.

T. H. PENNINGTON

Food for ruminants

Chemistry and Biochemistry of Herbage. Edited by G. W. Butler and R. W. Bailey. Vol. 1: pp. xx+639; vol. 2: pp. xiii+455; vol. 3: pp. xii+295. (Academic: London and New York, October 1973 and January 1974.) Vol. 1: £12; \$36; vol. 2: £8; \$25; vol. 3: £6; \$17.

THESE volumes provide for the first time a comprehensive source of information on the chemistry and biochemistry of herbage defined as plant material excluding seeds or roots grown for feeding herbivorous animals.

Chapters 1-11 deal mainly with organic constituents of plants, free and bound amino acids, amines and ureides; proteins and nucleic acids; non-structural carbohydrates; structural carbohydrates; lipids; plant phenolics; lignin; alkaloids; cyanogenetic glycosides and glucosinolates; chlorophyll, carotenoid pigments and sterols; forage saponins. Most chapters follow a similar pattern describing chemical nature, classification, extraction from tissues and estimation of these compounds, amounts present in plants and their component parts, biosynthesis and amounts of components, for example amino acids in proteins and fatty acids in lipids.

Perhaps it would have been more logical to include chapters 1 and 2 of volume 2 on vitamins, and water in herbage, in volume 1, and chapters 12 and 13 in volume 1 on mineral composition of herbage and nutritional aspects of soil ingestion by grazing animals in volume 2, along with four chapters on mineral absorption and composition of herbage, mineral biochemistry of herbage, organic acids and their role in ion uptake, and cycling of minerals in ecosystems.

Commendably, a good deal of attention has been given to linking treatment of subject matter, for example in the above chapters on minerals, and in chapters on physiology of light utilisation

by swards, growth senescence and stress, and organic reserves and regrowth. Further chapters in volume 2 deal with the biochemistry of photosynthesis, symbiotic nitrogen fixation by legumes, the nitrogen cycle of pasture ecosystems and genetic variation in herbage constituents, this last chapter making a fitting end to volume 2 and prelude to volume 3, in its consideration of problems encountered in breeding improved varieties and emphasis on the need to evaluate results against animal performance.

The more applied chapters of volume 3 deal with conservation of herbage as hay and silage, leaf protein as an animal and human foodstuff, digestion of pastures by ruminants and other herbivores, the feeding value of herbage and its evaluation by laboratory methods, and limitation to the productivity of the herbage-fed ruminant that arise from diet, including the effects of toxic components and metabolic diseases.

Advanced students, teachers and research workers will find here an invaluable source of information, for example on assessment of methods of extraction of compounds from tissues, and on areas where, because of paucity of information on herbage plants, recourse has been made to non-herbage tissues, thus pointing to the need for further research. Examples of this are the description of biosynthesis of lipid components in spinach, chlorella and bacteria, detailed studies of isolated cellulose from wood and commercial fibres, the need to assume that *in vivo* formation and inter-conversion of carbohydrates in leaves and stems are probably similar to those in seeds and tubers, and the belief that protein synthesis in plant cells is essentially similar to the mechanism worked out for bacteria and mammalian cells. Attention is constantly focused on the need for further research, for example, in comparing information available on temperate and tropical herbage and in the search for methods of measuring the nutritive value of herbage.

An attractive feature of the work is that agronomists and plant breeders will find useful biochemical treatment of several subjects such as ensilage and ruminant digestion, biochemists will find limitless avenues for research, while the ruminant, the ultimate beneficiary, is frequently brought into the picture. A worthwhile attempt, attended by considerable success, has been made to bridge the all-too-wide gap which frequently separates pure biochemists and workers in agricultural science.

On the whole the work is readable and, bearing in mind the diverse nature of topics covered in what are, for the most part, review chapters with appropriate lists of references, a reasonably even standard of treatment is achieved.

A good deal more attention could have been given to some topics however. The vitamins, for example are covered in only 11 pages, with no mention of important modern aspects such as vitamin D metabolites and the tocotrienols, and little consistency in description of symptoms of deficiencies of individual vitamins. Again, although commendable efforts have been made to take examples from many countries, this has not always been achieved: in chapter 27 all tables of hay composition are taken from the United States.

The volumes are attractively produced and the text contains few typographical errors. Minor blemishes noted in volume 2 include reference to "some mineral, possibly pectin" (page 110) and an obvious omission on page 119 (par. 2). Arrangements of some tables could also be improved.

W. M. ASHTON

NMR for enzymes

Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems. By Raymond A. Dwek. Pp. xviii+395. (Clarendon: Oxford; Oxford University: London, December 1973.) £8.

During the past ten years many attempts have been made to apply nuclear magnetic resonance (NMR) techniques to problems of biological interest and the subject has reached the stage when a comprehensive textbook is required to summarise and appraise the advances in this area. Thus Dr Dwek's book is opportune and is all the more welcome because this first comprehensive text on the subject is also a very good book.

In the chapters devoted to protein spectra, protein-ligand binding and the use of paramagnetic probes in such systems, the book indicates the extent to which NMR spectroscopy can be used to obtain three-dimensional structural information in enzymes, to identify functional groups involved in enzymatic reactions and to investigate the role of metal ions and coenzymes in enzyme systems. Dr Dwek's book attempts to provide a balanced view of the usefulness of the NMR technique in some of these areas by considering both its possibilities and limitations.

Adequate background NMR theory is given to allow for the understanding of the many applications considered. Emphasis is given to the theory of relaxation, exchange and paramagnetic shifts and relaxation because many of the more successful applications rely heavily on these aspects of magnetic resonance. Fortunately, Dr Dwek's research interests are in these areas and this is evident in his authoritative coverage of relaxation effects related to paramagnetic metals and spin-label

probes.

The book is well illustrated throughout with clearly labelled diagrams (although the labelling on the AMX spectrum in Fig. 2.14 is incorrect).

It is a pity that the chapter on protein NMR spectra was not introduced with a detailed section on amino acids and peptides. The problems encountered in trying to determine conformational information for small peptides would have put into perspective Dr Dwek's statement that "in principle, it should be possible to obtain from the spectrum the complete structure of a protein in solution".

The chapter on carbon-13 studies neglected to indicate the potential for studying strongly bound ligands in slow exchange (using ^{13}C enriched ligands to overcome the protein background spectrum) and also the advantages of studying ^{13}C relaxation times.

These are however relatively minor criticisms of an excellent book which can be recommended to anyone interested in learning more about enzyme systems.

J. FEENEY

Echo technology

Radar, Sonar and Holography. By Winson E. Kock. Pp. xvii+140. (Academic: New York and London, December 1973.) \$9.50; £4.45.

THIS book is intended to introduce high school and college students to the technology of radar and sonar, the principles of holography, and the impact of holography on echolocation. Its explanations successfully avoid the use of mathematical concepts or formulations. The opening chapters introduce waves, coherence, interference, diffraction, near and far fields and zone plates, radiators and colimators. But the author does not, for example, derive the diffraction beam-width of a slit from interference between its left and right halves, or explain the Rayleigh distance as the intersection of the near-field projected aperture and the divergent far-field beam.

Next the book outlines the principles of echolocation, and gives examples of conventional radars and sonars. There is, however, no mention of surface ducts, self or radiated noise, reverberation, or the uses of Doppler in sonar. There is a chapter on Doppler radar, but the Doppler effect is not explained. Though no reference is made to normal pulse-Doppler radars, dual-frequency systems are mentioned—without indicating that their Doppler effect is determined by the difference frequency.

Next the book introduces monochromatic optical, acoustic holograms, lucidly explained as a generalisations of the zone plate. This leads on to synthetic-aperture side-scanning aircraft radars, which are well described by analogy

to sequentially-generated holograms. The basic commonality of different configurations could, however, with advantage, have been explained in terms of reciprocity and symmetry. The use of coherent optics to synthesise the high-resolution near-field radar picture is clearly delineated.

Thereafter the author introduces some rather dubious material: he implies that large-aperture real arrays are a special case of synthetic-aperture systems, and have become possible only with the advent of holographic processing. He suggests that the number of elements in a given array aperture can be reduced without loss. (A reduced hologram can cover the same total picture, but only with an appropriately reduced resolution and/or contrast.) He fails to equate the gain from a synthetic-aperture end-fire system to that from a conventional coherent Doppler integrator; indeed there is no reference to the equivalence of coherent integration in the space and time domains. After recognising the equivalence between moving target indication (that is velocity null generation) and end-fire directivity (that is, rearward directional null generation), the author spoils it by stating that MTI is restricted to targets moving directly towards the radar.

The book describes pulse compression—but fails to distinguish between power and energy, or to indicate how 'chirp' frequency modulation is handled in practice. The elegant technique of 'zone-plate' amplitude modulation for optical pulse compression is explained, but not the more usual pseudo-random modulation and correlation detection. In discussing phased-array systems, the author extols holographic techniques for receiver beam forming, without reference to the range dimension (echo delay), within-pulse scanning, real-time processing, or adaptive beam steering.

Though open to such criticisms in detail, the book is very readable and well illustrated. It gives clear concise explanations of some modern concepts which are not well covered in popular technical literature. There are a few slips in proof reading, editing, history and theory—but probably no more than usual.

I feel the link between holography and echolocation has been overemphasised. I would also have opted for a less catholic coverage of 'with-it' technology and assorted applications, to make room for more explanation of physical principles and practical constraints. But this may merely reflect my preference for a book aimed at a somewhat more sophisticated readership. The author has probably met his own objectives and those of his senior high school readers well enough.

RALPH BENJAMIN

matters arising

Calibration of the radiocarbon time scale

SIR,—The recently published paper by Clark and Renfrew¹ on calibration of radiocarbon dates is the latest in a series on this topic²⁻⁵ by archaeologists who are rightly concerned by the uncertainties now involved in interpretation of data. Central to those papers has been the attempt to 'smooth' the bristlecone calibration curve of Suess⁶ to negate 'kinks' and thereby ease interconversion of radiocarbon and calendar ages. Here I sound a cautionary note on such practices and question the validity of the smoothing methods. There is no dispute here over the detailed statistical techniques invoked by the various authors. The issue in question is the overworking of experimental data. There is little doubt that the methods employed are justified neither by the quality of the ¹⁴C results nor by the present understanding of ¹⁴C geochemistry.

The key issue is centred on the vital difference between the absolute accuracy and analytical precision of the ¹⁴C measurements which comprise the bristlecone pine calibration curve. It is important to consider the inequality of these parameters. The error terms reported with ¹⁴C data are derived essentially from the statistical ¹⁴C counting error alone. To assess the real accuracy of the measurements, however, requires an answer to each of the following questions:

(1) Is the ¹⁴C level of wood grown at an altitude around 8000 feet indicative of concentrations at sea level and at all other latitudes?

(2) Is the ¹⁴C content of wood fractions other than the cellulose component necessarily a precise measure of atmospheric ¹⁴C levels during wood growth?

(3) Can experimental unknowns, such as counter behaviour, contamination effects, human error, standardisation and half life uncertainty, be totally neglected? Furthermore, are systematic differences between ¹⁴C laboratories possible?

To each of these questions there can, as yet, be no ideal answer. Comment can, however, be made:

(1) At the extreme altitudes where the Bristlecone Pine grows, the ambient cosmic ray neutron flux is approximately one order of magnitude higher

than at sea level. Since these neutrons are the source of ¹⁴C through the reaction $n + {}^{14}\text{N} \rightarrow {}^{14}\text{C} + p$, it is feasible that bristlecone pine wood could be non-representative of normal organic material. Whether through *in situ* ¹⁴C production or variable atmospheric mixing, bristlecone pine ¹⁴C levels could experience localised effects. Furthermore, there is already preliminary evidence that atmospheric ¹⁴C levels may be latitudinally dependent. Thus Jansen⁷ and Lerman *et al.*⁸ have observed depletions of 0.5% to 2% in ¹⁴C concentrations of southern relative to northern hemisphere wood.

(2) It has been shown with fair certainty that some chemical components of wood can be transported between tree rings⁹. Cellulose is chemically the most inert constituent of wood and, to minimise cross contamination, should be selected for precise ¹⁴C analyses. Perhaps due to the limited size of samples, the major American laboratories engaged in time scale calibration do not take this precaution.

(3) It would be unrealistic to imagine that either systematic differences between laboratories or occasional freak results within one laboratory do not occur. There are many potential causes of such deviations.

The factors discussed here cannot be quantitatively assessed at present because of incomplete evidence. Only future research can clarify the situation and, for this reason, independent calibrations of the radiocarbon time scale, such as now being developed at Queen's University, Belfast, are of major significance. In the meantime a realistic approach to the available ¹⁴C data is essential. In other words, besides the analytical error reported for each bristlecone pine ¹⁴C analysis, there exists a further major error term to be considered. At present we cannot quantify it. Nevertheless, in view of the geochemical aspects of the factors mentioned, it would be unwise to attribute a degree of absolute accuracy less than $\pm 2\%$ (2σ) to the bristlecone calibration curve (unless the archaeological specimen in question is a pinewood sample from an American mountain top and is pretreated and analysed by Suess). A 1% error in ¹⁴C content corresponds to a radiocarbon age error of 80 yr so that, including reported analytical errors, it seems at present unjustified to attribute an accuracy of less than ± 200 yr

to the calibration curve. In essence then the curve should be imagined not as a line but as a probability band of considerable spread (400 yr). Through this approach a realistic interpretation can be performed. Admittedly, the value of the calibration curve is severely reduced but this is the real situation which no mathematical manoeuvring should obscure.

Finally, I note that the simple approach of Suess, in adjoining ¹⁴C data by hand, is in many respects preferable to smoothing operations by computer. Our need at present is for awareness of the limits of available data and for an increased availability of such results. Smoothing operations, in their role of rendering the experimental data remote, are contrary to this requirement. In addition, given the major error terms inherent in the curve, there is little reason for sophisticated mathematics. Kinks may well exist. The real existence of one such anomaly is beyond dispute¹⁰. It is questionable therefore whether at this time it is objective to smooth them out.

So I urge patience and restraint. Calibration, if performed at all, cannot yet be as quantitative as would be desired. There can be no justification for the delusion that major uncertainties do not exist. Over interpretation of the calibration curve for archaeological purposes is, for the time being, unwarranted.

Yours faithfully,
M. S. BAXTER

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SIR,—Clark and Renfrew¹ state that "the hypothesis of a common calibration curve for the bristlecone pine and Egyptian data over the time period 3100 BC to 1800 BC is not contradicted and suggestions to the contrary are not well founded". But according to Fig. 2 of ref. 1, all but two of the 14 Egyptian data points for the period 2200 BC to 1800 BC indicate ¹⁴C dates that are less recent than indicated by the curve shown for the bristlecone pine, on average by one or two centuries. Consequently the statistical conclusion is difficult to accept; this viewpoint was put to one of the authors of ref. 1 by McKerrell at the 1973 Oxford Archaeometry Symposium, in support of his contention² that the bristlecone pine calibration is not consistent with the Egyptian data; but no explanation of the apparent paradox was then offered.

A close inspection of the figure gives an explanation. In the period 2100 BC to 1900 BC there is a substantial difference between the bristlecone pine curve and the tree ring data points that were used for the statistical analysis; this difference is in the same sense as for the Egyptian data. So the analysis by Clark and Renfrew cannot be held to contradict McKerrell's contention.

The tree ring data were taken from the unpublished thesis of Houtermans³ and comparison of these with the data points shown by Suess on the bristlecone pine curve⁴ confirms that there are differences between the two sets; since both sets originate from the same measurements one must presume that corrections have been applied to one set which have not been applied to the other. In the absence of other indications it seems reasonable to accept the published data rather than the unpublished. It is also relevant that using either of the bristlecone pine curves put forward by the Pennsylvania⁵ and Arizona⁶ radiocarbon laboratories (which incorporate the Suess data with later measurements) the Egyptian ¹⁴C dates shown for ~ 2000 BC are still significantly less recent than bristlecone pine ¹⁴C dates, though by not quite so much.

Yours faithfully,

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¹ Clark, R. M., and Renfrew, C., *Nature*, **243**, 266 (1973).

² McKerrell, H., *Proc. prehist. Soc.*, **38**, 286 (1972).

³ Houtermans, J. C., thesis, Univ. Berne (1971).

⁴ Suess, H. E., in *Radiocarbon Variations and Absolute Chronology* (edit. by Olsson, I. U.), Plate I (Wiley, New York, 1970).

⁵ Michael, H. N., and Ralph, E. K., *Proc. 8th Int. Conf. on Radiocarbon-dating, Lower Hutt City*, (edit. by Rafter, T. A., Grant-Taylor, T.), 28-43 (R. Soc. New Zealand, 1973).

⁶ Damon, P. E., Lond. A., and Wallick, E. I., *Proc. 8th Int. Conf. on Radiocarbon-dating, Lower Hutt City*, (edit. by Rafter, T. A., Grant-Taylor, T.), 45-59 (R. Soc. New Zealand, 1973).

DRS CLARK AND RENFREW REPLY: We agree with many of the points made by Baxter and in a paper now in the press¹ discuss the substantial statistical problems involved in constructing an adequate calibration curve from the data available. But the construction of a calibration curve was not, as Baxter seems to imply, the purpose of our recent paper². Instead an evaluation was sought of the general applicability of the bristlecone pine data and their associated radiocarbon dates. The radiocarbon dates obtained from dendrochronologically dated bristlecone pine samples will match radiocarbon dates from historically/archaeologically dated Egyptian samples only if satisfactory answers could be given to his three questions: a precise equivalence would give exactly the independent corroboration which he is seeking. In fact, although the two sets do not contradict within the limits of their associated errors, the match is an inexact one because of the magnitude of those associated errors.

There should be no question here of overworking experimental data. The procedure used was to present, apparently for the first time, a formal analysis of the consistency of the determinations from different laboratories, working always through a consideration of the measurement errors involved (both as reported by the laboratories themselves, and as established from replicate analyses). Is this not in fact the only acceptable way of comparing two independent data sets?

The geophysical problems which Baxter indicates are not new, and will only be answered by statistical analysis of existing data and by the gathering of new data in such a way that it will be susceptible to statistical analysis. He is certainly right to stress that the appropriate form of the calibration curve is not yet clear, and that an error of perhaps ± 200 yr is to be associated with the curve³.

Aitken is correct that the data presented by Houtermans and by Suess are not in precise agreement: we chose to use the former because they were more recent and did not have to be read from a graph. Aitken's reference to Fig. 2 of our paper is not, however, to the curves used by us to compare the bristlecone pine and Egyptian data, but to Suess's original curve presented there for purposes of comparison only. If the data points of Fig. 2 are compared with

the curves derived from those data, there is no contradiction. Consider, for example, the least-squares straight line. If the hypothesis (of a common calibration curve) is correct, one would expect roughly half of the data points to lie below this line. Inspection of Fig. 2 shows that, between 2200 and 1800 BC: (i) 5 of the 14 Egyptian points are below this line; (ii) 10 of the 19 bristlecone pine points are below this line.

Such a distribution of the 14 Egyptian points is not unusual; the probability of five or fewer points out of 14 being below the line is about 0.20 (from the Binomial distribution), so that there is no reason to doubt on this basis that the Egyptian points satisfy the same calibration relationship as the bristlecone pine data. The same argument could be applied to the entire interval from 3000 to 1800 BC. Figure 2 shows that 23 of the 52 Egyptian points lie below the fitted straight line, whereas 27 of the 57 bristlecone pine points lie below this line. One could hardly expect to get much closer to 50% than that.

It remains our conclusion that, on the basis of the available data considered, and within the error limits discussed (themselves unfortunately wide), there is no contradiction between the Egyptian and bristlecone pine data. This is an important conclusion since for some parts of prehistoric Europe the calibrated radiocarbon chronology differs by as much as 2,000 yr from archaeological chronologies put forward before the application of radiocarbon dating. At the same time, as Baxter rightly stresses, the error limits are large, and much more work will be needed before the form of the appropriate calibration curve will be known with precision.

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¹ Renfrew, C., and Clark, R. M., *Archaeometry*, **16**, (1974, in press).

² Clark, R. M., and Renfrew, C., *Nature*, **243**, 266 (1973).

³ Burleigh, R., Switsur, V. R., and Renfrew, C., *Antiquity*, **47**, 314 (1973).

Na⁺ transport defect in cystic fibrosis

SIR—Using the technique of ³H-ouabain binding to isolated cells Quissell and Pitot concluded that the Na⁺-K⁺ ATPase functions normally in fibroblasts from cystic fibrosis patients¹. However this finding may not be very relevant to the Na⁺ transport defect occurring in this genetic disease.

The Na⁺ transport abnormality in cystic fibrosis seems to be confined to exocrine glands². It is unknown at present whether the defect resides in an active or passive transport step of the cation. At least with isolated sweat

gland tissue³ and erythrocytes⁴ from cystic fibrosis patients, Na⁺-K⁺ ATPase activity has been found to be normal. However a decrease in the ethacrynic acid-sensitive efflux^{5,6}, a Na⁺ exchange mechanism⁵, has been reported in cystic fibrosis erythrocytes^{6,7}. These findings suggest that there is no fundamental abnormality in the Na⁺-K⁺ ATPase enzyme in the disease, but there may be a defect in an ethacrynic acid-sensitive transport step. Therefore in order to obtain a better understanding of the cation transport defect one should measure the binding of labelled ethacrynic acid rather than ouabain.

It is possible, however, that there is no basic abnormality in the mechanism of Na⁺ transport in cystic fibrosis but in the action of agents regulating this process^{8,9}. Membrane transport of this cation is controlled by hormones and cyclic AMP in a tissue specific manner¹⁰. Failure of a Na⁺ transporting system in exocrine glands to respond to a specific hormone or cyclic AMP could explain why the defect is confined to these organs. Isolated model systems like fibroblasts and erythrocytes might not therefore show up this defect.

Yours faithfully
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Drs Quissell and Pitot respond:

Due to the pathophysiology of cystic fibrosis, conclusions derived from data obtained from intact patients with the

disease or from biopsy material obtained from CF patients must be carefully scrutinized. Several factors, such as anoxia, infection, malabsorption, liver and pulmonary involvement, and the nutritional and hormonal status of the patient complicate the interpretation of these data.

The Na⁺ transport defect could be closely related to the genetic defect or the Na⁺ transport defect could be secondary. The small salivary glands and the eccrine sweat glands are affected by the electrolyte defect in cystic fibrosis¹¹ but the submaxillary and parotid glands secrete normal levels of sodium^{2,12}.

The decrease in the ethacrynic acid-sensitive efflux observed in cystic fibrosis erythrocytes⁶ is difficult to interpret¹³. Lapey and Gardner⁷ observed a normal sodium efflux in heterozygotes and in younger females with cystic fibrosis. The mechanism of action of ethacrynic acid on the overall membrane transport process is not known. In fact, the inhibitory process may involve one or several intracellular metabolic events rather than events on the membrane *per se*^{14,15}. Ethacrynic acid does not seem to bind specifically to the membrane but to all cellular fractions¹⁶. Cystic fibrosis patients seem to have normal kidney function except for a lower metabolic clearance rate and a higher plasma aldosterone level which is probably due to the excessive loss of sodium in the sweat and a subsequent decrease in the intravascular volume¹⁷.

If an alteration in the hormonal response in cystic fibrosis exocrine tissue is observed, the alteration could be intimately related to the primary defect in the disease but the alteration could be secondary to its pathophysiology.

Careful evaluation will be required but the results should be very interesting.

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obituary

Sir Charles Dodds

SIR CHARLES DODDS, Bt., biochemist and physician, who died on December 16, 1973, was at the time of his retirement in 1965 the senior professor in the University of London, having been elected at the age of 25 to the newly created Courtauld Professorship of Biochemistry at the Middlesex Hospital Medical School. Two years later, when the Courtauld Institute of Biochemistry was built he was its first Director, an appointment that he was to occupy with the highest distinction for 38 years. His

originally tiny staff of three graduates expanded steadily so that due to his stimulating and inspiring leadership the Institute has acquired a fine reputation for both teaching and research.

Edward Charles Dodds was born on October 13, 1899, brought up in Darlington and moved with his parents to London, where he was educated at Harrow County School and the Middlesex Hospital Medical School, supporting himself by various scholarships and later by tutorials. He was entirely a 'Middlesex man', who never seemed to wish to work elsewhere. Very early he

showed his life-long interest in hormones, developing a method which proved useful for preparing insulin for the Hospital.

When they were both 25, he and F. Dickens wrote *Chemical and Physiological Properties of the Internal Secretions*. Dodds meanwhile was in charge of the Chemical Pathology and pioneered in this country the colorimetric methods of analysis which Folin had recently introduced in the United States. He was called in during the illness of King George V and, then aged 29, received the MVO. With George Beaumont he produced *Recent Advances in Medicine*

which ran to 14 editions. In more recent years, he was one of the first to introduce automation into clinical biochemical analysis in this country.

Dodds own research reached its highest point in the discovery of the highly potent artificial oestrogenic hormone, diethyl stilboestrol and its relatives. With Wilfrid Lawson, he explored the weak activity which they had detected in a range of simple non-steroidal compounds, until they found a highly active by-product arising in the demethylation of the simple phenolic ether anethole. They discovered the structure and synthesis of the compound, in work with Sir Robert Robinson, in 1938. Stilboestrol was even more active than the natural hormone, could be manufactured in quantity, and was effective when given orally. In addition to its value in gynaecology, it proved, following the lead of Charles Huggins, to be the first oral drug to be able to control a form of cancer—carcinoma of the prostate—even when the disease was disseminated.

The honours and decorations conferred on Dodds over the years occupy a whole column of Who's Who, but among these were his Knighthood in 1954 and Baronetcy 1964. He was elected FRSE. 1941; FRS 1942 (Vice-President 1957-9); FRCP 1933 (Harveian Librarian later) and President of the Royal College of Physicians in 1962—unique for a professor of biochemistry to be so honoured. He held honorary degrees from universities all over the world, was widely travelled and had lectured—though this did not come easily to him—in many countries. He succeeded Lord Horder as Chairman of the Scientific Committee of the Cancer Research Campaign; an excellent chairman, he was in constant demand in this capacity, serving on numerous Government and other scientific committees. His medical school owes to him the revolutionary reorganisation of the teaching of biochemistry, and of chemistry, which he insisted should be integrated subject; he built up a devoted staff of gifted teachers.

Though somewhat shy by nature, he was never aloof with his colleagues, always ready to discuss their problems, tempering his shrewd advice with a nice touch of humour. It delighted him when he became Master of the Society of Apothecaries in 1947-9, and nothing pleased him better than entertaining his many friends to good food and good wine, on which he was something of an authority.

His marriage in 1923 to Constance Elizabeth Jordan of Darlington was a happy one and he never seemed to his close friends quite to recover from her death in 1969. Their only son Ralph succeeds to the baronetcy.

Announcements

Corrigendum

In the article "Heavy ion transfer reactions in nuclear astrophysical processes" by T. W. Conlon (*Nature*, **247**, 268; 1974) the third paragraph from the end should read as follows. "A study of the α -transfer reaction $^{12}\text{C}(^{16}\text{O},^{20}\text{Ne})^8\text{Be}$ at energies in the region of the Coulomb barrier has been made possible by the development of a particle identification system based on position-sensitive counters mounted in the focal plane of a Buechner spectrograph. Preliminary results indicate that significant amounts of ^{20}Ne are produced with respect to ^{24}Mg , which results from the principal compound nucleus channel (Table 2)."

In the same article, the following corrections should have been made; para. 1, line 12 should read "corresponds to the transfer of an α particle, . . ."; para. 10, line 5, "available for later evolutionary . . ." and the last column in Table 1 should be " Q optimum (MeV) corresponding to ($E_{\min}$ to $E_{\max}$)".

International meetings

May 14, **Our Environment—Museum or Habitat?** (Lisa Strange, The Ship and Boatbuilders' National Federation, 31 Great Queen Street, Kingsway, London WC2B 5AD)

May 15, **The Possible Role of Anti-Tumour Immunity in Radiotherapy** (The General Secretary, British Institute of Radiology, 32 Welbeck Street, London W1M 7PG)

May 16, **Friction, Wear and Lubrication** (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX)

May 19-22, **Second International Banff Conference: Man and His Environment** (Miss E. M. Buchanan, Conference Supervisor, Division of Continuing Education, The University of Calgary, Calgary, Alberta T2N 1N4)

May 20-23, **The Impact of Offshore Oil Operations** (Mrs Jill de Wardener, The Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR)

May 20-23, **5th National Convention of the Royal Australian Chemical Institute** (Professor B. J. Ralph, School of Biological Technology, University of New South Wales, P.O. Box 1, Kensington N.S.W. 2033, Australia)

May 20-24, **2nd International Symposium on Biotelemetry** (ETHZ Turnen and Sport, Labor. für Biomechanik, Plattenstrasse 26, CH-8032 Zürich, Switzerland)

May 20-25, **7th World Congress on Occupational Safety and Health** (World Congress Secretary, Ansley House, Dublin 4, Ireland)

May 21-23, **Rubber Processing 1974** (Martin J. Quinlan, Publications Officer, The Institution of the Rubber Industry, 4 Kensington Palace Gardens, London W8 4QR)

May 21-24, **The Science and Technology of Powders and Fine Particle Systems** (Institute for Fine Particles Research, Summer School, 1974, Laurentian University, Sudbury, Ontario, Canada P3E 2C6)

May 22-24, **Workshop/Symposium on Compliance with Therapeutic Regimens** (David L. Sackett, Room 3H2, McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8S 4J9)

May 23, **Structure and Function of Intracellular Junctions** (R. C. Hughes, National Institute for Medical Research, Mill Hill, London NW7 1AA)

May 24, **The Future of the Polytechnics** (The Secretary and Registrar, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex SS1 2JY, UK)

May 29-31, **28th Annual Symposium on Frequency Control** (Commander, US Army Electronics Command, Attention: AMSEL-TL-MF, (J. R. Vig), Fort Monmouth, New Jersey 07703)

May 29-31, **26th Annual Brookhaven Symposium in Biology: Processing of RNA** (John J. Dunn, Biology Department, Brookhaven National Laboratory, Upton, New York 11973)

May 29-June 1, **Electronic Properties of Oxides: Applications and Science** (Professor R. W. Vest, School of Materials Engineering, Purdue University, West Lafayette, Indiana 47907)

May 29-June 2, **5th International Congress of Cytology** (Alexander Meisels, Secretary General, 5th International Congress of Cytology, 1050, Chemin Ste. Foy, Quebec 6, PQ, Canada)

May 31-June 1, **International Symposium on Jaundice** (Gordon Awade, Executive Director, The Canadian Hepatic Foundation, 65 Queen Street West, Toronto, Ontario, Canada)